TECHNICAL MEMORANDUM 19

PATHOGENESIS OF ANTHRAX - A PROGRESS REPORT

NOVEMBER 1962

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FOREWORD

". . . what we do in the way of experimentation depends to a large measure on what we think . . ." (W. M. Stanley*)

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* On the nature of viruses, cancer, genes, and life—a declaration of dependence. R. A. F. Penrose, Jr., Memorial Lecture. American Philosophical Society, April, 1957.

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ACKNOWLEDGMENTS

ANIMAL EXPERIMENTATION

In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society of Medical Research.

ABSTRACT

The dynamics of the host-parasite interaction after challenge with anthrax was studied by developing a procedure for survival experimentation that cannulated the thoracic and right lymph ducts. After aerosol challenge bacilli were observed in the lymph as early as nine and usually before 16 hours post-challenge and prior to observations of bacilli in the blood. Most bacilli are retained in the reticulo-endothelial system (RES) through an incubation period until the preterminal septicemia occurs. This stage is characterized by a logarithmic increase in numbers of bacilli and an increasing concentration of toxin. The doubling rate in the septicemic stage and number of bacilli/milliliter of blood at death are constants in each host species; however, immunization reduces the terminal number. Four different experimental approaches show that the rhesus monkey infected with anthrax dies of toxin. Two unevaluated tests give early indication of anthrax infection, namely cholinesterase inhibition and a cytoplasmic effect for guinea pig tissue cell sheets. A live vaccine as a booster following antigen results in a tremendous increase in resistance to challenge. Antibiotic treatment with mice work shows clearly that penicillin is the antibiotic of choice. Streptomycin, aureomycin, and terramycin have value; chloramphenicol is of little value. A mathematical model of pathogenesis of anthrax has been developed.

SUMMARY

The dynamics of the host-parasite interaction after challenge with anthrax was followed in several species of animals. For survival experimentation a procedure was developed for cannulating the thoracic and right lymph ducts (and, in which, simultaneous cannulation of the jugular vein was possible). The basic procedure was to enter the mediastinum by opening of the chest by bisection of the sternum. The course of the disease in the rhesus monkey after aerosol challenge followed a regular, predictable pattern as summarized below.

After aerosol challenge, bacilli were observed in the lymph as early as nine hours and usually before 16 hours post-challenge and prior to their observation in the blood. Spores were never observed in the lymph or blood. The build-up of bacilli in the lymph is not a gradual increase in numbers, as is the terminal septicemia. Rather the number of bacilli changes rapidly from zero to a maximum value, then remains relatively constant through the course of the disease. Thus, it appears that, as bacillary growth overcomes one node, it flows to and is retained by the next successive node until the last node is overcome. Bacilli then enter the blood in high volume at the right or thoracic duct and are disseminated by the blood throughout the body. An incubation period characterized by a low, irregular bacteremia follows. The incubation period depends on the (a) size of the dose, (b) virulence of the pathogen, and (c) resistance of the host. Organisms increase in numbers in the body principally in the fixed tissues of the reticulo-endothelial system (RES) until a preterminal septicemia is observed. During this incubation period antigenetically active proteins are produced and toxin has been identified in lymph as long as 11 hours before death. The logarithmic increase of bacilli in the blood and increasing concentration of toxin in the body fluids are the outstanding features of the preterminal septicemia. The doubling rate (apparent generation time) in the blood stream is constant, regardless of (a) whether or not the host is immunized, (b) the size of inoculating dose, (c) the virulence of the strain, or (d) whether virulence-enhancing chemicals are used. A doubling rate of 45, 115, 55, and 65 minutes that is characteristic for each species was determined for mice, rats, guinea pigs, and monkeys, respectively, whereas the terminal concentrations for the respective species are $10^{7.0}$, $10^{5.9}$, $10^{8.3}$, and $10^{6.5}$ bacilli/milliliter of blood of nonimmunized animals. Ther terminal number can be changed by immunization, strains of organism, strain of animal, and duration of infection. In the guinea pig, a reduction from $10^{8.3}$ to $10^{6.0}$ has been observed when guinea pigs were immunized with serial injections of protective antigen followed by a booster dose of live vaccine. With one strain, V116, the guinea pig has a two-times higher terminal number of bacilli in the blood than with the V1b strain. With V1b strain of organism, the Fischer 344 rat has only $10^{3.0}$ bacilli/milliliter at death; the NIH black rat has $10^{5.9}$. With this species, a low terminal number is related to susceptibility to toxin. The terminal number may vary

with time of incubation and, in the rhesus monkey, animals dying at about 48 hours following aerosol challenge have an average of about $10^{7.5}$ organisms/milliliter of blood; those dying 96 to 120 hours post-challenge have only $10^{5.0}$ bacilli/milliliter.

Four different experimental approaches show that animals infected with anthrax die of toxin. We have shown that (a) toxin is present in the terminal blood of virtually all monkeys dying of anthrax; (b) for as long as 11 hours before death, toxin can be demonstrated in the lymph of monkeys infected either via aerosol or intradermally, and the concentration of toxin increases steadily from the time of its first detection until death; (c) sterile in-vitro-produced toxin injected intravenously in sufficient amounts kills monkeys unless death is prevented by specific antiserum; and (d) death with symptoms of toxemia occurs within two hours after injection of 10^{11} spores, whereas 10^{10} spores or less require 20 or more hours of growth in vivo to cause death.

Monkeys dying of anthrax have the classical symptoms of anthrax in domesticated and experimental animals, as well as man. No symptoms have been observed that are useful in indicating an early infection. Preliminary and unevaluated observations indicating early infection are (a) the whole blood cholinesterase activity is completely inhibited after infection and at least 24 hours before death, and (b) a cytoplasmic effect for guinea pig tissue cell sheets can be demonstrated in thoracic lymph collected 18 hours and less before death of intradermally infected monkeys. This earliest detection was six hours before bacilli were observed in the lymph, seven hours before toxin could be detected by the Fischer rat test, and 16 hours before bacilli were observed in the blood. Since the tissue culture cells respond to in-vitro- and in-vivo-produced toxin in different ways, it is possible that different responses are being measured. The blood cell ratios change significantly, and sedimentation rate decreases in blood of rats given sublethal doses of toxin.

The extent to which immunization affects resistance to challenge can be accurately expressed as an immunity index, I, which represents the $\log_{10}$ change in dose required to cause the immunized host to respond (die) in the same time as the control host. The immunity index of guinea pigs injected intraperitoneally with five serial injections of protective antigen (Belton-Strange) is 3.2; a booster dose of antigen increases I to 6.0. If the booster dose is live vaccine instead of protective antigen, I = 8.0 and, if a second booster dose of vaccine is given, I = >11.0. In the latter case, it is impossible to kill guinea pigs with doses of one billion spores given parenterally. With guinea pigs, the increase in aerosol dose required to infect immunized animals does not closely parallel the dose required by parenteral response, and is significantly lower. No association between serum antibody titer and the immunity index (resistance to actual challenge) was detected in guinea pigs or rats immunized to any one level; however, when all levels of resistance were considered, a positive association was apparent.

The agar diffusion test was more variable than the immunity index, especially at relatively low or intermediate levels of protection. Immunization with protective antigen is effective in changing the spore dose required to initiate infection. However, if (a) the spores are germinated, (b) vegetative cells are used, or (c) egg yolk is added to the challenge dose of spores, the dose required to infect parenterally is reduced to nearly that required to infect nonimmunized hosts with normal dormant spores. As reported above, the terminal concentration of bacilli/milliliter of blood is significantly lower in immunized than in nonimmunized hosts and this suggests that immunization with protective antigen may not be toxin-neutralizing. Alternate interpretations suggested are that, during the presepticemic stage, relatively greater amounts of toxin are produced in immunized hosts than in nonimmunized hosts, or increasing the host's immunity also increases the host's sensitivity to toxin. Compared with Vlb, three strains of anthrax are refractive to immunity, i.e., to a dose level of about 2.7 logs (500 times) following parenteral challenge of guinea pigs.

Initial work has been completed to separate and concentrate the three known components of anthrax toxin by column chromatography and electrophoresis.

Techniques were developed to count the bacilli in the whole animal, as well as in selected organs and the blood, in order to better understand the dynamics of infection and treatment.

Treatment work to date has been with mice. When given in a single injection, penicillin can cure infection later post-challenge than can streptomycin, aureomycin, terramycin, or chloramphenicol. Penicillin, streptomycin, aureomycin, and terramycin are about of equal value if treatment is begun early and continued through at least four days. Chloramphenicol is without value in treating this disease if treatment is initiated four hours post-challenge or later. Nonimmunized mice given protective antigen at the same time as they were challenged parenterally die from a significantly lower dose than the dose that killed mice not given protective antigen.

A mathematical model of the pathogenesis of anthrax was developed to help in quantitating host responses so that we may safely bridge our understanding of this disease from experimental animals to man.

Immediate program plans are stated.

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FIGURES

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I. INTRODUCTION

In our 1959 report entitled "Anthrax in the BW Program"[1] we observed that new approaches to the problem of determining the dose required to infect man by aerosol challenge were required. At that time new facts on anthrax had been published but no re-evaluation of this disease considering these facts had been made. Publications of particular importance were the observations of Ross[2] on passage of spores from the lung alveoli into the body, the identification of anthrax toxin by Smith et al,[3] and proof of the presence of toxin in the terminal blood of guinea pigs dying of anthrax. In addition, certain new techniques were noted, including marked advancement in model dynamics. After Trnka et al[4] showed that maximum growth of anthrax in sheep occurs in the lymph 10 to 16 hours prior to initiation of a septicemia, we suggested that cannulation of the thoracic duct might be a means of early detection of systemic anthrax infection. The basis of our earlier report was that the new facts forced reconsideration and questioning of every problem and previously formed view on the pathogenesis of anthrax. We realized at the time of our first report, as well as now, that the challenge of man with virulent anthrax spores is only a possibility. It is not even a probability until laboratory animals can be cured of generalized anthrax.

In spite of our recognition of the importance of developing a treatment that will cure generalized anthrax, work in this area has just recently been initiated since an understanding of the course of disease was not available. Lacking the basic research, treatment could be approached only on an arbitrary basis, using current philosophies and techniques that had been adequately proved to be incapable of curing generalized anthrax. Early detection, as well as a cure late in the course of disease, might substitute for a deeper understanding of this disease. These achievements, too, are apt to follow complete knowledge of pathogenesis. We concluded that only a broad approach to pathogenesis, emphasizing the quantitative aspects of disease, would furnish the information required to allow us to accomplish our objectives.

We have studied anthrax in naturally resistant and susceptible hosts and hosts resistant by immunization, when challenge is with high or low doses of either virulent or lowly virulent strains and by four routes of challenge. We have been interested in determining the inter-relationships among variables and levels of these variables and learning how to change the interaction between pathogen and host and, thereby, the course of disease. A partial report of our work has been made in several publications; however, this Technical Memorandum is the first progress report of work on pathogenesis. All data have been presented in order that others may independently evaluate it. As our work progresses, it becomes evident that generalized anthrax is an orderly, predictable disease; consequently, the interruption of its course, i.e., treatment, after controlled infection appears feasible.

II. MATERIALS AND METHODS

A. GRADED RESPONSE-VIRULENCE TEST

This test requires a dose high enough so that all or essentially all animals respond. We have used death as the response and selected the dose to give response at the desired time. Graphically, the dose-response curve over a range of several logs is a straight line. This is true for all species of animals tested, both normal and immunized (Figure 1). It may be noted that the dose-response curve extends into the quantal area continuing the linear dose-response relationship into the partial response area. The method and comparative variance of the graded and quantal tests have been treated more fully elsewhere.[5-6]

B. IMMUNITY INDEX

From the graded dose-response curve, the immunity index is readily developed. Immune animals require a higher dose to respond in the same time as the nonimmunized hosts. The log of the difference in dose required to cause the same time of response is the immunity index, I. This relation-ship is represented graphically in Figure 2. The immunity index has been discussed more fully[5] and its use illustrated[6] elsewhere.

C. QUANTITATIVE ESTIMATION OF BACILLI/MILLILITER OF FLUID BY DIRECT OBSERVATION

The quantitative estimation of bacilli/milliliter of blood or lymph usually is done by dilution plate count. The number in the sample is known 18 to 24 hours later. We have used the direct counting method of Keppie et al[7] in which a standard volume of fluid is spread over a one-cm^2 surface. The bacilli are fixed, killed by immersion in ten per cent formalin, and stained. The number of bacilli for a known area is determined, and a standard curve of number of bacilli/microscopic field vs number of bacilli/milliliter is developed through the range of 10^4 to 10^9 bacilli/milliliter. Regression of direct count on plate count is r = 0.99. The count by direct observation requires about 45 minutes. This method has a lower limit of practical observability at 10^4 bacilli/milliliter. Counts of any desired accuracy (any standard deviation) can be made by varying the number of microscopic fields (area) counted. With our standardized set-up, we observe 144 fields routinely for counts of 10^4, 10^5, or 10^6 and fewer fields when the count is higher. Normally, dilution plate counts also are made, frequently on a four-hour interval.

The direct observation method is rapid, requires 0.1 milliliter or less of blood, may be a permanent record, and allows the experimenter to determine the exact stage to which the disease has progressed in each animal.
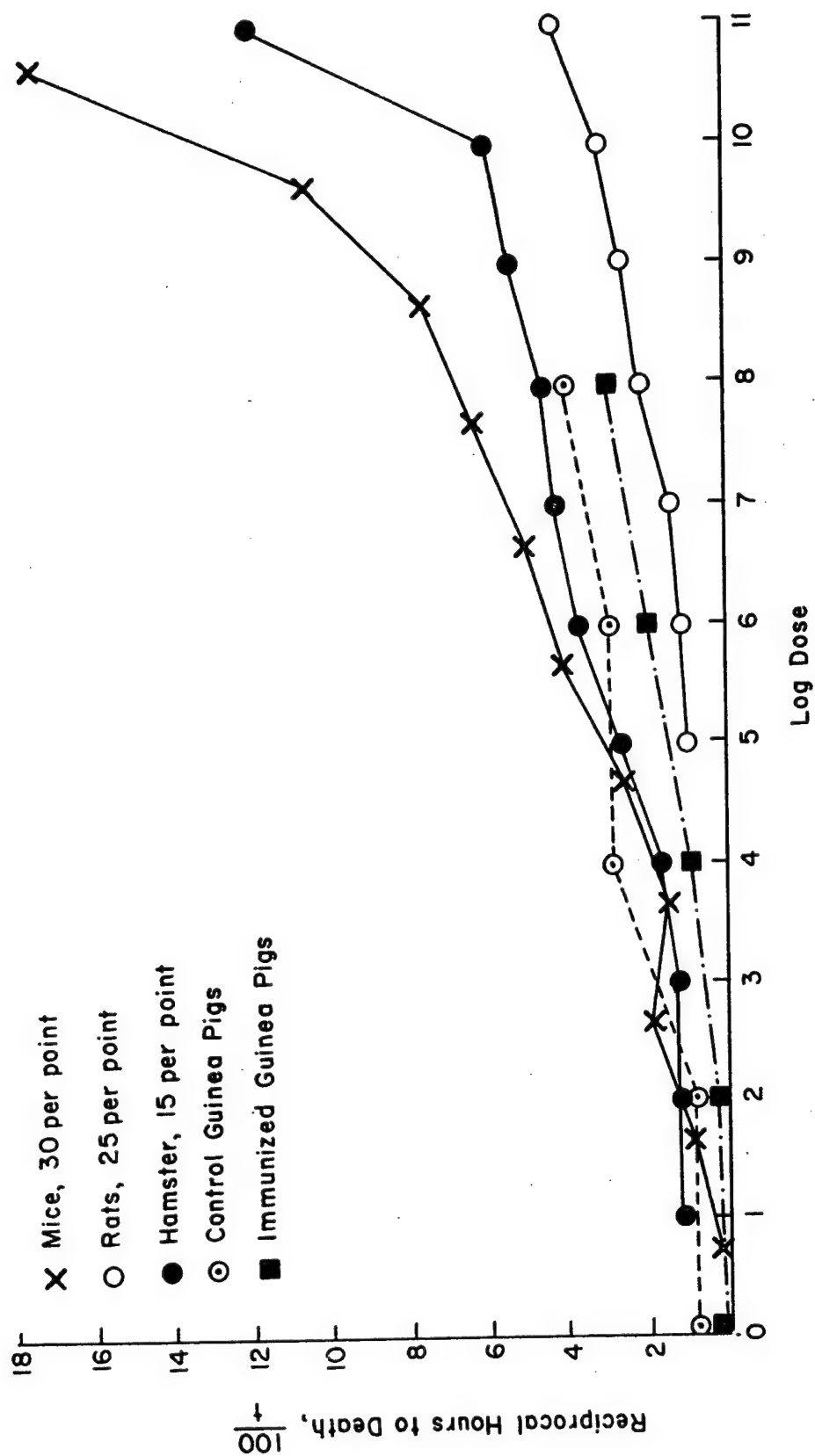
Figure 1. Relation of Mean Reciprocal Time-to-Death of Animals with Dose of B. anthracis Spores.

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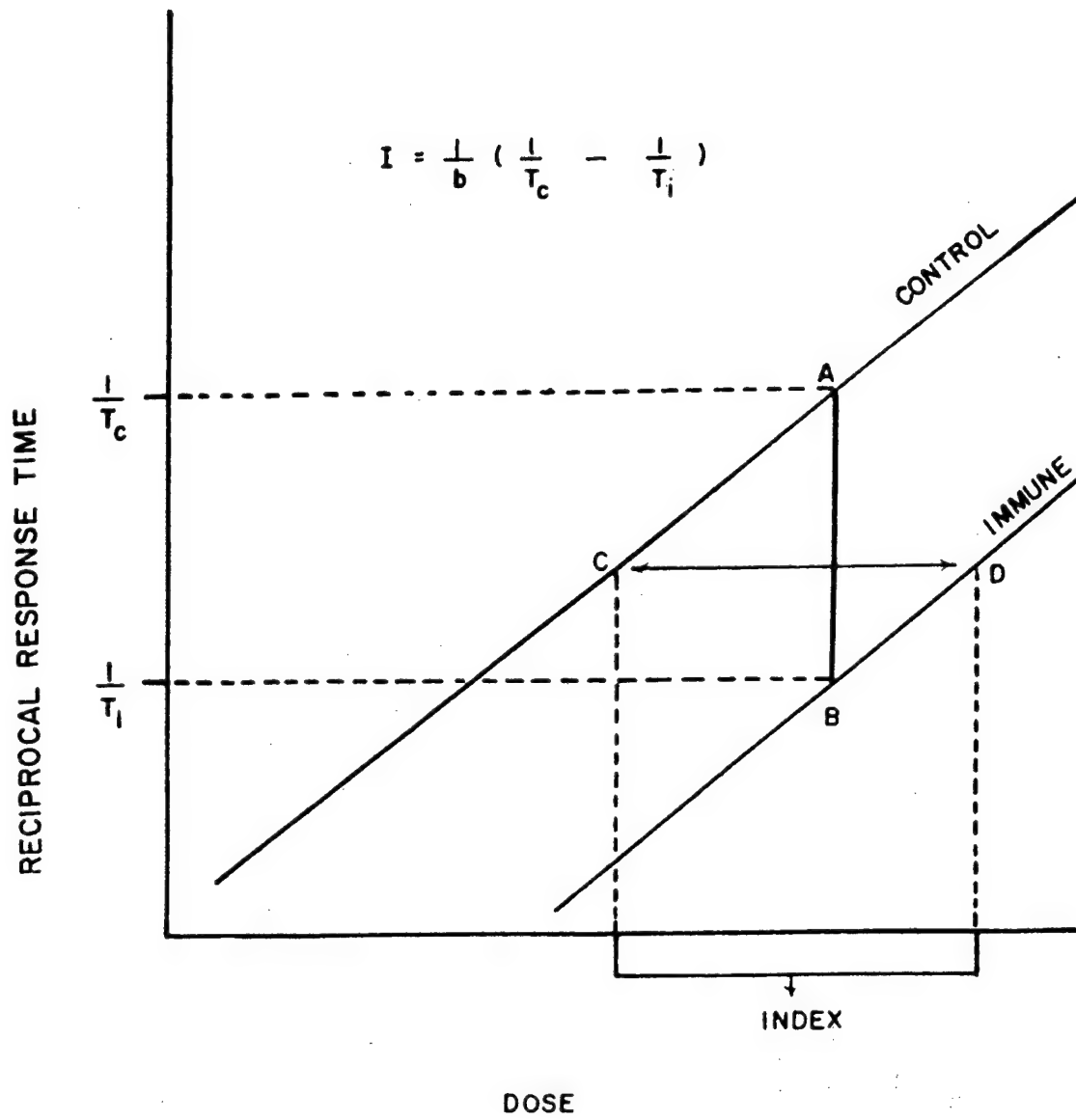
Figure 2. Immunity Index.

D. CANNULATION OF THE THORACIC AND RIGHT LYMPH DUCTS AND OF THE JUGULAR VEIN OF THE RHESUS MONKEY

A full report of this technique is·presented in the Appendix and by Hodges and Rhian.[8] In addition, a movie[9] was made of this work. In brief, exposure of the thoracic and right lymph ducts and jugular vein is achieved by entry of the duct through the superior mediastinum by bisection of the sternum. The lymph ducts may be cannulated if they form a common duct before entering the jugular vein. The cannulae are placed so that they emerge just posterior to the ear by tunneling under the skin. Simultaneous cannulation of the jugular vein allows a cannula to be placed in the heart. When cannulation of the lymph ducts is not desired, cannulation of the jugular vein is achieved by a simpler operation.

Before the monkey recovers from anesthesia, it is placed in a holding chair so that it is prevented from reaching the cannulae protruding from the back of its head. The lymph flows continuously and is collected as desired. The blood cannula and a solution of either glucose or protein hydrolyzate are connected to a three-way stop-cock. Blood may be sampled when desired by inserting a syringe on the third outlet of the stop-cock. After a blood sample, the cannula is always back-washed with sterile solution. Glucose and protein hydrolyzate are given on a regular schedule to prevent dehydration and hypoproteinemia of animals from which lymph is being collected. This method of blood and lymph sampling allows samples to be taken on any schedule by·one person and without exciting the animal.

III. RESULTS

Our proposed hypothesis for pathogenesis of anthrax[10] is, in many ways, similar to the stages observed for pathogenesis of other bacterial and viral diseases. We consider that the following infection events occur with in- halation anthrax, and we will discuss the experimental work under the several visualized successive events of infection and pathogenesis. The successive events are:

(a) Deposition of the spore in the alveoli.

(b) Transport of bacilli to lymph stream by macrophage and movement to lymph node.

(c) Multiplication in lymph and node.

(d) Entrance into blood stream at right and thoracic lymph duct and collection of bacilli in reticulo-endothelial system (RES).

(e) Release from reticulo-endothelial system and entry of bacilli into lymph or into capillaries, with re-entry into blood stream and establishment of secondary multiple foci of infection.

(f) Septicemic increase of organism and toxin preterminally.

(g) Death of the host.

(h) Hematology, symptoms, and gross pathology.

A. DEPOSITION

We have not studied factors changing deposition of spores in the lung. The surface on which spores may be deposited is vast. Data from Best and Taylor[11] indicate that in man the total epithelial surface of the lungs is in the order of 70 square meters, of which about 55 are respiratory. The spore surface is not smooth but rather rough and more or less echinate. The spore demonstrates hydrophobicity and, presumably, will have a charac- teristic electrical charge. The sporangium may or may not remain attached to the anthrax spore. Until stimulated to germinate, the spore probably remains as inert as a carbon particle. Several studies indicate that about 20 to 25 per cent of the inhaled spores remain in the lungs of animals exposed to clouds of "single" "dry" spores. Harper and Morton[12] showed that monkeys are variable in respect to both retention of particles and sites of deposition, due to differences in rate and depth of breathing and to mouth breathing. Barnes[13] showed that 70 per cent of the retained spores were recovered in the stomach one-half to one hour after exposure. Detrick wartime work[14] and that of Henderson et al[15] indicate that further removal

of spores from the lungs is extremely slow. The aspects of increased deposition and increased removal of deposited spores from the lungs must ultimately prove a very productive research area and lead to a more complete understanding of the disease.

B. TRANSPORT TO LYMPH STREAM BY THE MACROPHAGE

Ross[2] demonstrated that spores deposited in the alveoli are ingested by motile phagocytes that migrate through the undamaged epithelium and enter the lymph stream. The spore-bearing macrophages move to the tracheobronchial nodes. Germination probably occurs after phagocytosis. Evidence obtained by parenteral and aerosol challenge indicates only a very few cells are needed to initiate the disease. A wartime estimate[14] of the most probable number to establish anthrax in guinea pigs by the respiratory route was two spores. Following intraperitoneal (IP) challenge, 25 spores treated with egg yolk caused 100 per cent death of the NIH black rat in 36 hours. Barnes[13] estimates that only 0.1 per cent of the spores deposited on the alveoli move into the lymphatics.

Parenteral virulence is enhanced by egg-yolk treatment of the spore or by germination and injection of either the germinated spore or the vegetative cell at the two-cell stage. Strains of B. anthracis of both low and high virulence are enhanced in virulence by egg yolk for the mouse, rat, immunized and nonimmunized guinea pig, and rabbit. The effect is maximum with strains of low virulence and highly resistant hosts and may change the dose required to establish disease as much as 10,000 times. Data to support these statements are reported by Rhian et al.[16] After egg-yolk treatment, there is development of a massive, early encapsulation, greater retention at site of deposition, and, perhaps, early or changed germination requirements. We have shown that egg-yolk treatment affects only the presepticemic course of disease, because in the septicemic stage, terminal number of organisms in the blood and the apparent doubling rate are the same in animals dying of anthrax, whether or not the challenge dose had been treated with egg yolk.

Early germination and encapsulation were demonstrated both in vivo and in vitro. Young rats were challenged intraperitoneally with and without egg-yolk-treated spores. One rat of each of the treatments was sacrificed each hour during a 12-hour holding period. Peritoneal fluid of each of the rats was examined microscopically. Germinated organisms were observed in both series, with and without egg yolk, after the first hour. In all remaining hourly sacrifices, organisms were observed only in the peritoneal fluid of animals inoculated with egg-yolk-treated spores. From the two-hour sacrifices until death, bacilli increased in the peritoneum and exhibited large capsules. At death, the peritoneal fluid contained a tremendous number of bacilli completely engulfed in capsular material. The presence of bacilli in the peritoneum of rats challenged with the treated spores suggests a high retention or active growth of organisms at the site of inoculation. Comparable results were obtained in vitro with whole blood

of rats, guinea pigs, monkeys, hampster, and man. Equal proportions of whole blood and spores treated with and without egg yolk were sampled every hour over a 24-hour period. Samples of each blood preparation were examined microscopically. Germination and outgrowth of bacilli occurred after the first hour, with large capsules becoming prominent in 8 to 12 hours and enlarging upon continued incubation. Germination or outgrowth could not be demonstrated with the nontreated spores.

The effect of egg yolk or of germination to reduce the dose required to establish anthrax by parenteral challenge does not carry through to the aerosol route. Rhian et al[16] showed that via the aerosol route, spores in egg yolk were less infective than spores per se. The mean doses and time-to-death response times for mice and guinea pigs were:

Host	Spores			Spores plus Egg Yolk		
	Dose	MTD	Mortality	Dose	MTD	Mortality
		Hour	%		Hour	%
Guinea Pig	$10^{4.63}$	67	91	$10^{4.93}$	254	41
Mice	$10^{4.08}$	107	50	$10^{4.38}$	255	27

When the amounts of solids (dextrin) in a spore suspension were increased over a ninefold range and the dose of spores administered by aerosol was held constant, Rhian et al[16] reported that infection from aerosols was decreased. The mean time-to-death increased significantly as the amount of nonspore solids was increased. The aerosol LD_{50} was directly proportional with the solids content.

Solids, %	LD_{50} Spores
0.55	13,400
1.67	18,800
5.0	162,200

Infection from suspension given by injection was not influenced by the solids content. The two explanations discussed by the authors were (a) that egg-yolk-treated spores germinated too soon, were inactivated by the phagocytic system and, therefore, could not initiate infection and (b) solids in the aerosol interferred with ingestion and/or transport of the spores by macrophages. Further studies on changing virulence by physical or chemical treatments are needed.

We have done a few tests to develop a chemical co-agent. In one test, we studied nine detergents, and in a second test, chlorides of five cations. These experiments were designed to test the premise that spores can be changed so that more are phagocytized by the motile phagocytes of the lung, resulting in a greater proportion being subsequently moved into the lymph stream or, alternately, that resistance of spores to destruction by phago-cytosis may be increased, resulting in a greater percentage of the phago-cytized spores establishing disease. Results were inconclusive; however, wartime work here[17] and at Porton[18] indicated that Tergitol enhanced viru-lence of anthrax when challenge is via aerosol.

The contrasting results for infection by different routes of challenge are of major importance to studies on infection and disease. If the observations reported are, in general, correct, it will be impossible and even misleading to use results based on parenteral infection experiments to predict results from aerosol exposure.

C. MULTIPLICATION OF BACILLI IN LYMPH STREAM AND NODE

Most of the drainage of the lungs is through the right lymph duct. Cannulation of this duct has been very difficult and all the four monkeys in which we were able to place a cannula were challenged via the aerosol route. All lymph that passes through the duct is collected; therefore, only that entering the collateral circulation channels enters the blood stream. Bacilli were found in the lymph of the cannulated right lymph duct as early as nine hours following challenge. In all cases, at about 16 hours many bacilli were observed. Although tested for, spores were never found. The build-up in the lymph is not a gradual increase but usually is a zero to maximum type. We interpret this to mean that bacilli entering the lymph are being filtered out or held completely by each successive lymph node. As the growth overcomes one node, it flows to the next and so on, until the last node is overcome and cells enter the blood. On this basis we have successive overflows and, depending on the sampling interval, we usually observe growth at a near maximum at the time of each overflow. Our obser-vations support Ross[12] in that the lymph is the route of entry of anthrax into the body.

With intradermal inoculation at a site drained by the popliteal node, observations on passage of organisms through the thoracic lymph are parallel with those above on the right lymph duct, that is, few or no organisms are observed during the 12- to 16-hour post-challenge, then large numbers are observed and the maximum number is reached within one to two hours after first observation of bacilli. This reaction might be compared to a continuous culture system. Lymph formation continues at a constant rate, bacilli are growing at a constant rate, and the outflow rate is constant; therefore, an equilibrium is reached and maintained. This is in contrast with the blood, where the septicemic stage builds up at a constant rate and is still increasing at death of the host.

D. ENTRANCE OF BACILLI INTO BLOOD STREAM AT RIGHT AND THORACIC LYMPH DUCT,
    AND COLLECTION OF BACILLI IN THE RETICULO-ENDOTHELIAL SYSTEM (RES)

The bacilli entering the blood from the lymph are removed by the highly
efficient RES to a level below that detectable by sampling of one or two
milliliters of blood and plating it on agar. The same observations apply
to challenge by the intravenous (IV) route. When 10^5 spores are introduced
intravenously, the number of bacilli/milliliter is below detection by our
sampling procedure at the 16-minute post-challenge sample. When 10^9 spores
are introduced intravenously, the number is reduced below a detectable
point in one to two hours; however when more than 10^9 spores are administered
intravenously, blood samples remain positive. By other routes of challenge,
it is normal to have few or no positive samples until the septicemia is
initiated, unless the dose is very high. When we do find positive samples
(a bacteremia), the observation of positive samples usually lasts only for
one sampling period, and the number is of the order one to ten cells/milli-
liter, occasionally 100 or greater.

This stage is considered to be a period of incubation in which many of
the bacilli produced are destroyed by the body defenses while others estab-
lished new foci of infections in the reticulo-endothelial system.

E. SECONDARY INFECTION AND INCUBATION

Observations have been recorded showing that anthrax bacilli can be
phagocytized and remain viable, as indicated both by multiplication of cells
while within the phagocyte and by egestion from the phagocyte and subsequent
growth of the bacillus. Regarding egestion, Oakberg[19] reports that, when a
macrophage in vitro contains between 32 and 64 organisms (Salmonella
typhimurium), the bacilli are released from the cell. During this period,
release of cells occurs with subsequent reinfections and establishment of
secondary and tertiary foci of infection. Our hypothesis of recycling of
bacilli is that most of the bacilli released from the RES and organs enter
the thoracic lymph system, circulate to re-enter the blood at the thoracic
duct, with the capillary circulation being minor. On this premise the
thoracic lymph should have a build-up of bacilli prior to its observation
in the blood. This requirement has been fulfilled in most cases when
challenge is by aerosol, intraperitoneal, or intradermal (ID) routes.
Since only the intravenous route tests the hypothesis without qualifications
and the single IV challenged monkey that also was cannulated did not meet
this requirement, more observations are being made. A final conclusion as
to the accuracy of this phase of the general hypothesis will be made when
new data are obtained following IV challenge. Data collected to date are
presented in the next section. In theory, if collateral circulation were
zero, all bacilli released from the reticulo-endothelial cells would be
collected by cannulation of the thoracic lymph duct and death would either
be prevented or the time-to-death extended significantly. There appears to

be some extension of time-to-death, but it is not a markedly significant one. We conclude, tentatively, that the number of bacilli entering the blood directly from the reticulo-endothelial system or by collateral circulation of the lymph is higher than predicted in the original hypothesis.

Apparently any tissue can become infected, although our knowledge of this aspect of anthrax is limited and mostly based on observations made after a septicemia has been observed or died. Keppie et al[7] have given the best data, namely that 84 per cent of the bacilli were in fixed tissue at six hours before the death of guinea pigs; at death, 72 per cent were in the blood. The Porton data and the data we present in the next section show that there is a remarkably constant inverse relationship between increasing level of bacilli/milliliter of blood and decreasing time-to-death. The fact that tends to be overlooked and requires emphasis is that, actually, there is a long incubation period, during which time, many bacilli are produced, so that by the time a septicemia does occur much damage due to toxin and other aggressions has occurred.

F. SEPTICEMIC INCREASE OF BACILLI AND TOXIN PRETERMINALLY

The preterminal phases may be discussed as two stages, septicemia and toxemia.

Each species and strain of animals appear to have a characteristic rate of development and final level of septicemia. We have observed the following constants:

With:	Doubling Time, min	Terminal Concentrate, org/ml
rat (NIH)	115	10^6
rat (Fischer)	115	$10^{3.5}$
mouse	45	$10^{6.9}$
guinea pig	53	$10^{8.3}$
monkey	65 (approx)	$10^{8.0}$ (approx)

The doubling rate of the septicemic stage in guinea pigs is not affected by any treatment we have tried. Treatments include immunization, egg-yolk treatment of the spores, virulence of strain, dose of inoculum, and route of challenge. Insofar as tested, the same is true for other animals. Only two treatments change the terminal level of guinea pigs, immunization, and strain of pathogen, while the strain also affects

terminal level of the rat. With immunization the level is lowered. We
have observed the following:

Host	Treatment	I Index	Terminal Concentrate, org/ml
Guinea pig	Control	0	$10^{8.3}$
Guinea pig	PA-5	3.2	$10^{7.7}$
Guinea pig	PA-5 + LV	7.5	$10^{6.0}$
Rat	Control	0	$10^{6.0}$
Rat	PA-5 + LV	4.0	$10^{3.2}$

The strain of organisms may affect the terminal concentration. Vlb and its
low virulent mutant, 30R, have the same terminal concentration as that al-
ready given. When challenge is with the V116 strain the terminal concentra-
tion is higher, about two times, from $10^{8.3}$ vs $10^{8.7}$. In an experiment
reported elsewhere[20] and as shown in Figure 3, the terminal level of bacilli
in the blood is reduced from 2×10^8 cells/milliliter in nonimmunized guinea
pigs to 5×10^7 in guinea pigs immunized with five successive injections of
protective antigen to an immunity index of 2.7 (500 times). The terminal
level was not changed when challenge was with 10^9 or 10^5 spores/animal or
when virulence was enhanced by egg yolk. The doubling rate (apparent gener-
ation rate) of 53 minutes (41- to 73-minute range) was not changed by im-
munization, dose, or egg-yolk treatment. Mean time-to-death of the respec-
tive treatments varied between 20 to 100 hours. We have since shown that
the terminal level of bacilli in the blood of animals immunized with protec-
tive antigen, followed by a booster of live vaccine, is reduced to 1/250th
of the control level, i.e., 10^6 cells/milliliter. In a comparison of the
Fischer 344 and the NIH black rat, we observed differences in the terminal
level attained between several ages and strains of rats. The slopes of the
regressions of concentration of organisms, computed from plate counts on
time against age of the host, were statistically significant at the 95 per
cent level. Slopes for 23-day NIH rats and for all Fischer rats were not
significantly different from zero. Terminal concentrations of organisms
per milliliter of blood, with standard errors of these values for all ages
of both strains, are presented in Table I.

The mean response times of the control and immunized animals challenged
with spores or egg-yolk-treated spores are shown in Table II. These data
illustrate the effects of increased virulence due to egg-yolk treatment and
the interaction of this effect with immunity when challenge is by the paren-
teral route. Challenge of control animals with egg-yolk-treated spores
decreases their response time from 63 hours to 47 hours or, as is shown at
the bottom of the table, by 25 per cent. It is also seen that the reduction
in resistance due to egg yolk is nearly three times as great—or 69 per cent
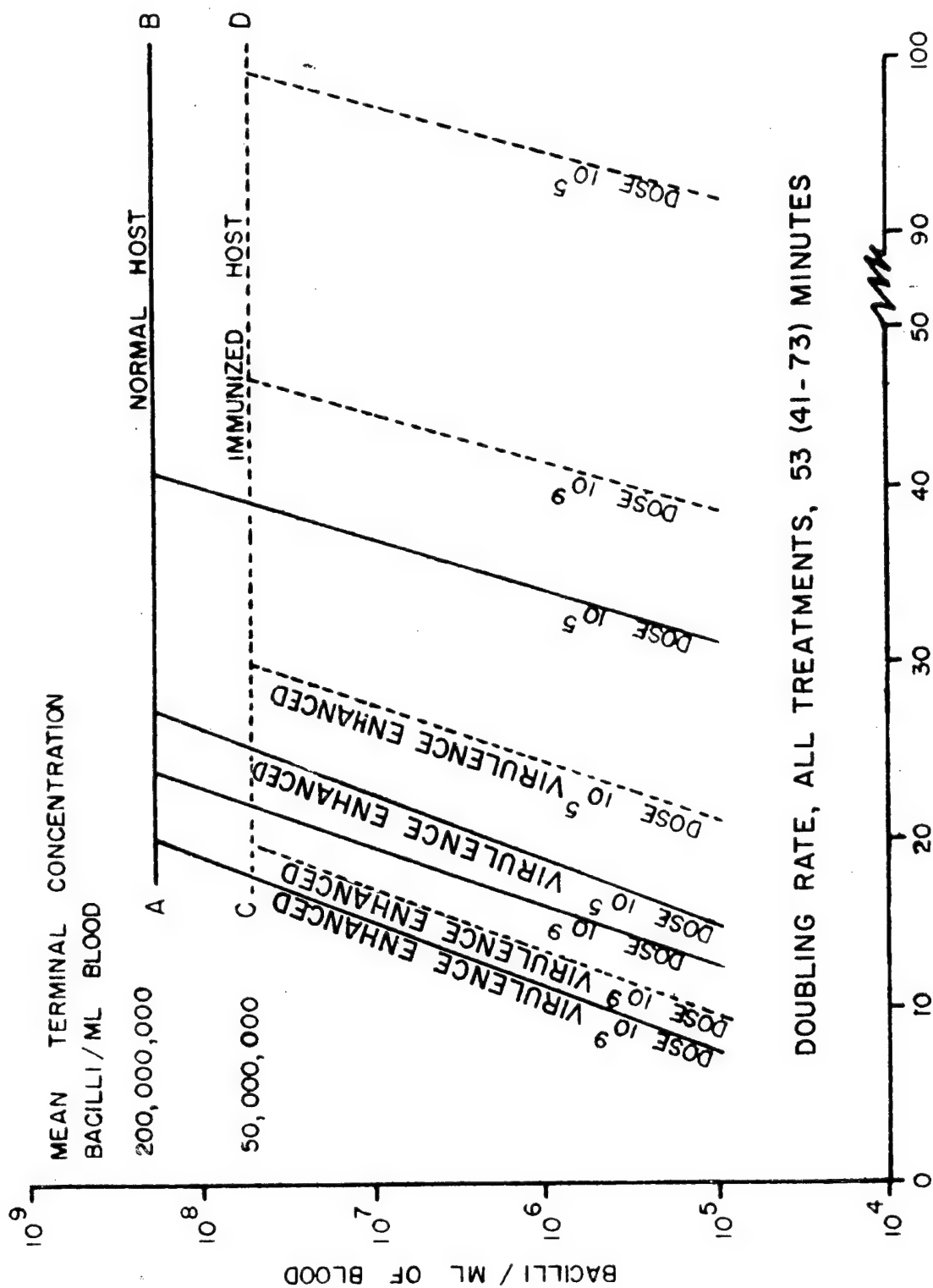
Figure 3. Time, Hours After Challenge with Virulent Spores.

TABLE I. ESTIMATES OF SLOPES OF SEPTICEMIA AND TERMINAL CONCENTRATION OF ORGANISMS IN THE BLOOD OF RATS BY TYPE AND AGE

Rat Strain	Age of Rat, days	Terminal Concentration,		Doubling Rate	
		Mean, log	Standard Error, log	Mean, log	Standard Error, log
NIH	76	6.3	0.26	0.17	0.06
	44	5.1	0.14	0.05	0.01
	23	5.0	0.44	0.37	0.30
Fischer	76	3.8	0.11	0.01	0.02
	44	4.2	0.11	0.02	0.01
	23	4.3	0.14	0.05	0.03

TABLE II. MEAN TIME-TO-DEATH BY SIZE AND TREATMENT OF CHALLENGE DOSE AND IMMUNIZING PROTOCOL

Immunization	Challenge Dose, log	Mean Time-to-Death, hours	
		Control Dose	Egg-Yolk-Treated Dose
Control	5	41	27
	9	22	20
	TOTAL	63	47
PA-5	5	101	26
	9	47	20
	TOTAL	148	46

Reduction in MTD Due to Egg-Yolk Treatment:

Control $\dfrac{63 - 47}{63} = 25.4$ per cent

PA-5 $\dfrac{148 - 46}{148} = 68.9$ per cent

in PA-5-immunized animals. It may be noted that control animals challenged with nontreated spores lived longer than immunized animals challenged with egg-yolk-treated spores (63 vs 46 hours). These data show that immunity against nontreated spores can be neutralized by the egg-yolk treatment. We have noted that this treatment of spores changes the germination dynamics, and the reduced dose required to establish anthrax in immunized hosts by the parenteral route is strikingly parallel to the earlier observations that cells at the two-cell stage from the spore, i.e., vegetative cells, are more virulent than spores when challenge is parenteral, and these effects carry through to immunized hosts. It appears, therefore, that immunization acts to increase the dose required to establish anthrax when the challenge material is spores, and immunization is relatively ineffective when the challenge organisms are germinated.

These data show another aspect of immunization that is significant in understanding the dynamics of anthrax. As stated earlier, the doubling rate in the septicemic stage is not affected by the treatments used in this experiment, including immunization with protective antigen; however, the terminal concentration of bacilli in the blood was four times higher in the nonimmunized animals than in the immunized hosts. These data indicate that the host defenses do not change during the final septicemic stage of this disease and, equally important, suggest that immunization with protective antigen is not toxin-neutralizing. Alternate suggestions in regard to toxin would be (a) during the presepticemic stage, relatively greater amounts of toxin are produced in immunized hosts than in nonimmunized hosts; and (b) increasing the host's immunity also increases the host's sensitivity to toxin.

In earlier reports,[1,10] we have indicated the desirability of following the establishment and course of anthrax when challenge is by the several possible routes. Data obtained following aerosol, intraperitoneal, intravenous, and intradermal challenge of the rhesus monkey are presented in the following paragraphs. All data collected are presented because the authors believe the variability of results will be as important when considering human challenge as will mean values.

In Figures 4, 5, and 6, data on the concentration of bacilli in the lymph and in the blood of monkeys challenged via aerosol are given. These figures may be inspected from the standpoint of (a) evidence for passage of bacilli in the challenging material into the body, (b) whether the lymph build-up occurs prior to the septicemia, and (c) evidence that organisms build up in the thoracic lymph prior to a septicemia. There is no evidence of bacilli passing directly from the lung into the blood since neither spores nor bacilli have been detected in the blood until at least nine hours post-challenge and after they first appear in the lymph. Shortly after this initial detection, the number of bacilli builds up rapidly over two to four hours to a very high number. The apparent generation rate during the build-up period is much lower than reasonable, based on in vivo observations of growth during the septicemic stage and in vitro rates in

Figure 4. Bacilli per Milliliter of Blood or Lymph of the
Rhesus Monkey Challenged by Indicated Route
and Dose of Virulent Vlb Anthrax Spores.
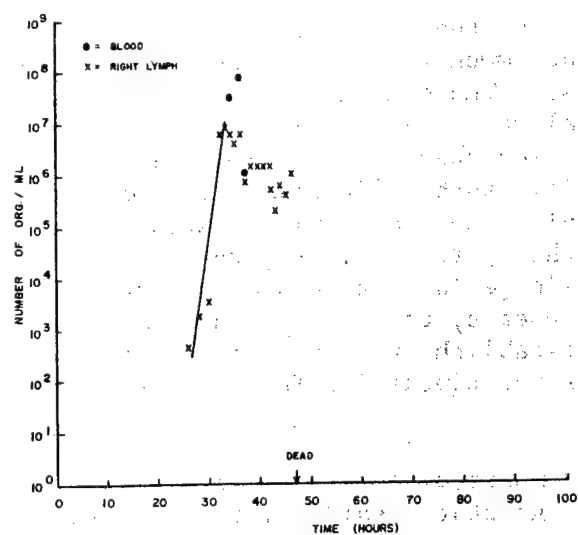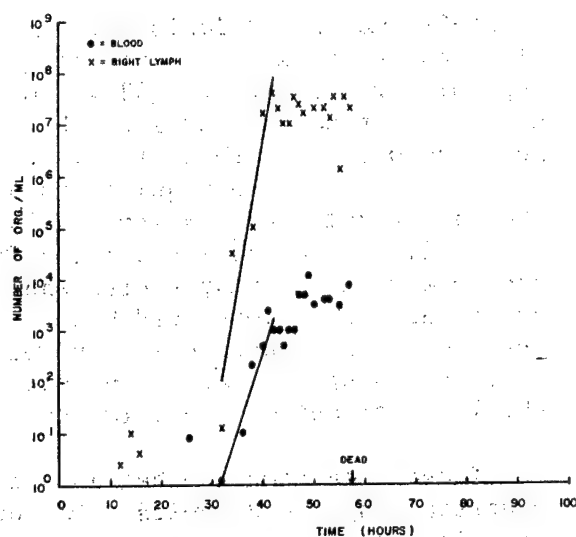
Figure 5. Bacilli per Milliliter of Blood or Lymph of the Rhesus Monkey Challenged by Indicated Route and Dose of Virulent Vlb Anthrax Spores.

Route   **AEROSOL**

Dose   **1.14×10^4**   **Spores**

Figure 6.   Bacilli per Milliliter of Blood or Lymph of the Rhesus Monkey Challenged by Indicated Route and Dose of Virulent V1b Anthrax Spores.

nutrient media. Bacilli are not demonstrable in the blood samples until after the build-up in the lymph. These observations may be interpreted to support Ross' observations of the route of infection. On the average, the bacilli in the lymph build up prior to the observation of a septicemia. However, individual variation and exceptions to this generalization have been observed. Finally, evidence of circulation of bacilli through the thoracic lymph is not definite and, at this time, this route must be viewed as a minor rather than a major route.

Similar data to that just presented for the aerosol route are presented for challenge by the intraperitoneal route in Figures 7 and 8; for the intravenous route in Figures 9, 10, and 11; and for the intradermal route in Figure 12. It is to be noted that the intravenous route is definitely an exception to any generalization that bacilli build up in the thoracic lymph prior to the observation of a septicemia. Normally, for all routes except intravenous, occasional bacilli may be observed for short periods in the blood prior to the septicemia. During the septicemia, the number of organisms/milliliter of blood increases logarithmically until death at an apparent generation time of 60 minutes. Death occurs typically when the terminal concentration is between 10^7 and 10^8 cells/milliliter.

As the disease progresses and, particularly at the time a septicemia is observed, a toxemia is detectable. Death of experimental animals is due to secondary shock and complications caused principally by toxin. The Porton work[3] demonstrating toxin showed that death will occur even if the body is sterilized of organisms early in the septicemia at a time when the number of organisms in the blood is low. The Porton workers could demonstrate toxin in the blood only terminally. Later, two groups at Fort Detrick[21-22] showed toxin present terminally in the blood of monkeys dying of anthrax. We believe that a knowledge of toxin is essential in understanding this disease and its treatment.

Quantitative assay for toxin was based on two procedures: The first, that of Beall et al[23] based on the susceptibility of Fischer rats to anthrax toxin, and the second, based on the agar diffusion technique described by Thorne and Belton[24] to measure the precipitation of antibody and antigen.

Preliminary studies suggest that monkeys infected with anthrax die of a toxemia. This inference is supported by observations from four aspects: (a) toxin is present in the terminal blood of monkeys dying of anthrax, (b) toxin increases in concentration in both the lymph and blood reaching a maximum at death, (c) sterile toxin alone causes death of the monkey and death may be prevented with specific antiserum, and (d) rapid death occurs in monkeys following an injection of 10^{11} B. anthracis spores. The observations to support these inferences are presented in the following paragraphs.

Figure 7. Bacilli per Milliliter of Blood or Lymph of the Rhesus Monkey Challenged by Indicated Route and Dose of Virulent Vlb Anthrax Spores.
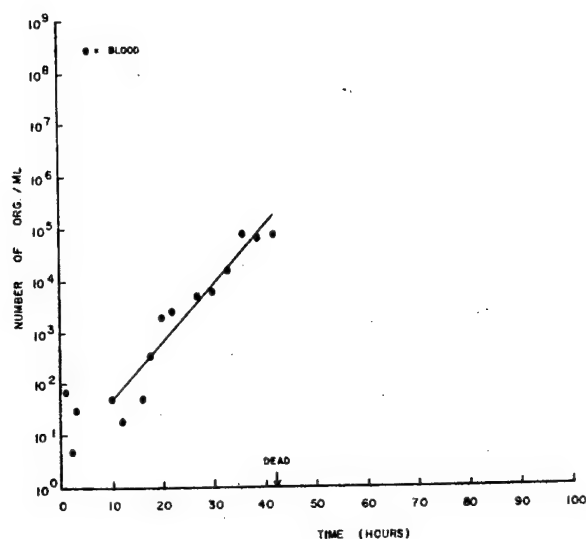
Route IP
Dose 1.16×10^7 Spores
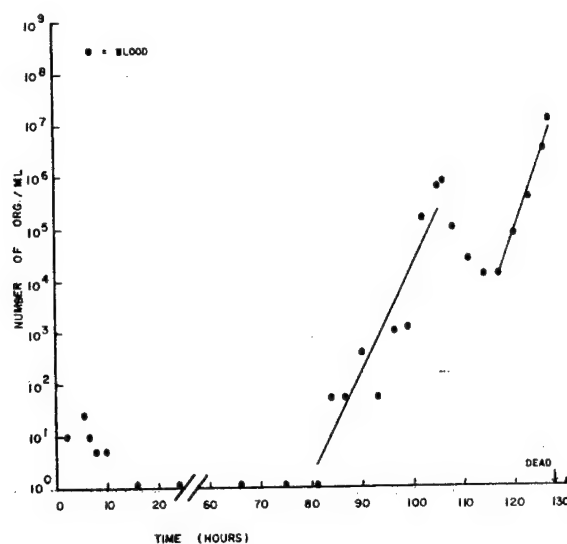
Route IP
Dose 9.28×10^7 Spores
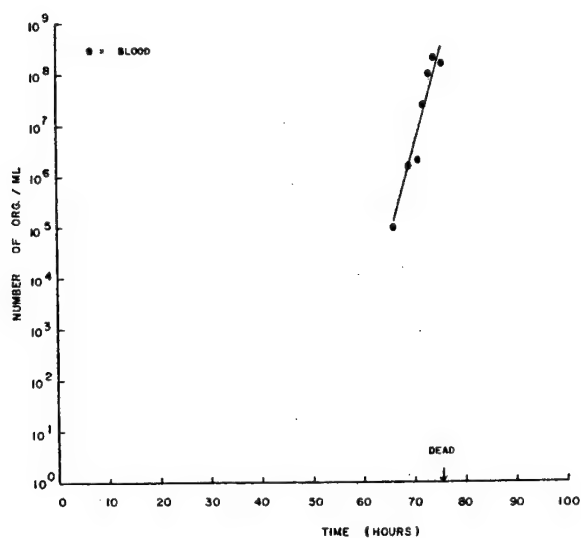
Route IP
Dose 1.14×10^5 Spores
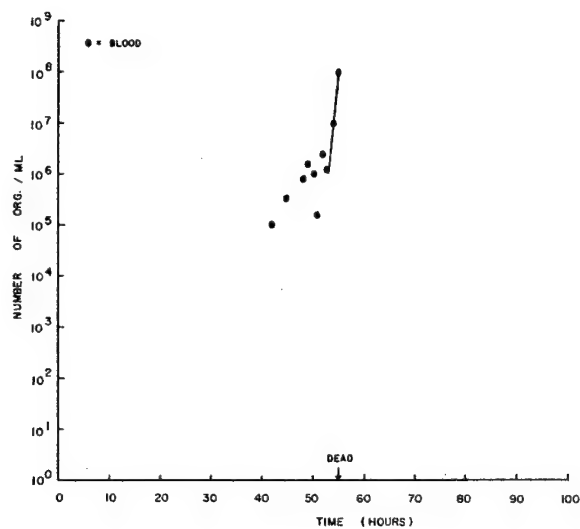
Route IP
Dose 1.14×10^5 Spores

Figure 8. Bacilli per Milliliter of Blood or Lymph of the
Rhesus Monkey Challenged by Indicated Route
and Dose of Virulent V1b Anthrax Spores.
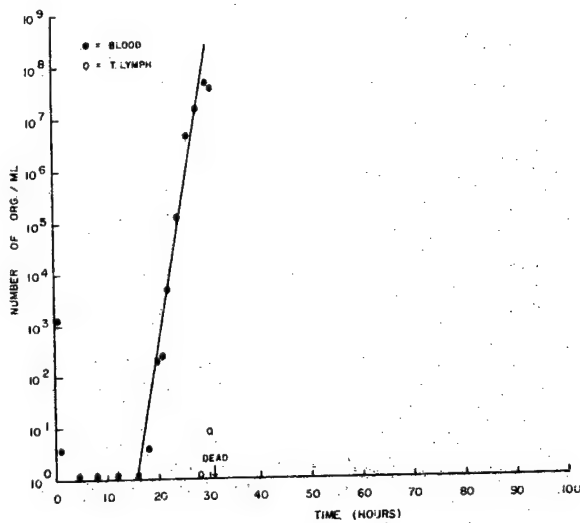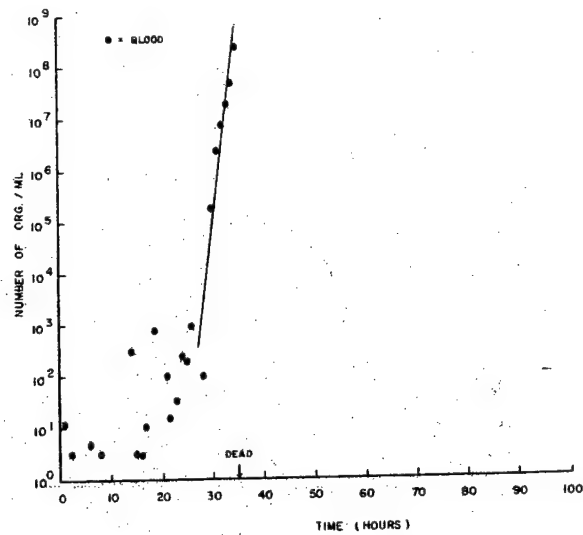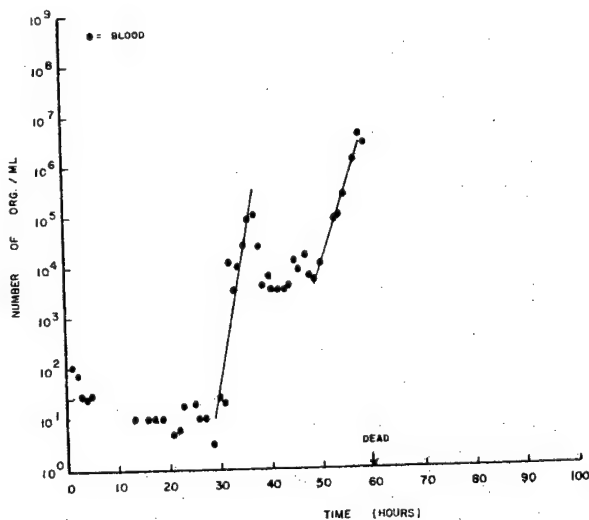
Figure 9. Bacilli per Milliliter of Blood or Lymph of the
Rhesus Monkey Challenged by Indicated Route
and Dose of Virulent Vlb Anthrax Spores.
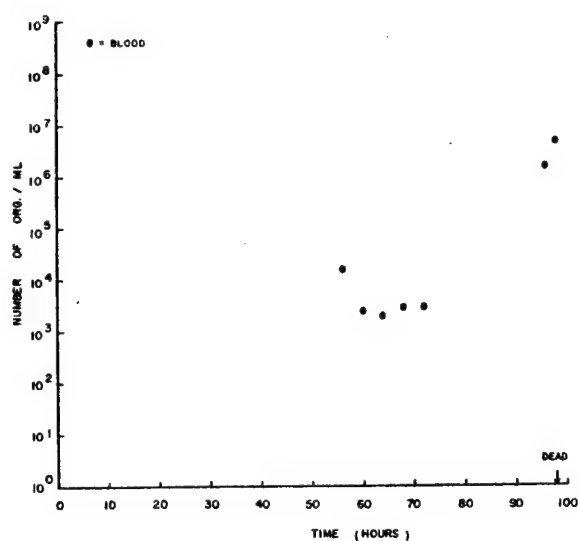
Figure 10. Bacilli per Milliliter of Blood or Lymph of the
Rhesus Monkey Challenged by Indicated Route
and Dose of Virulent Vlb Anthrax Spores.
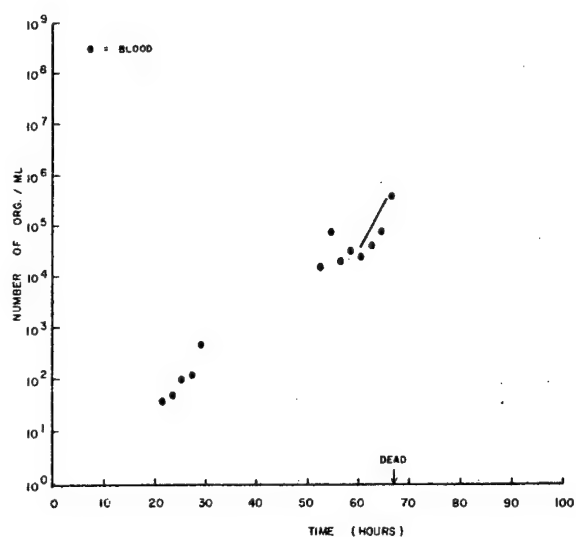
Route   IV

Dose 1.00×10^8 Spores
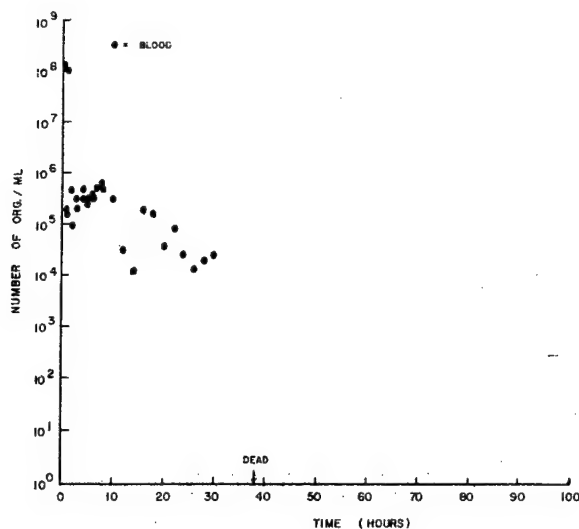
Route   IV

Dose 5.70×10^{10} Spores
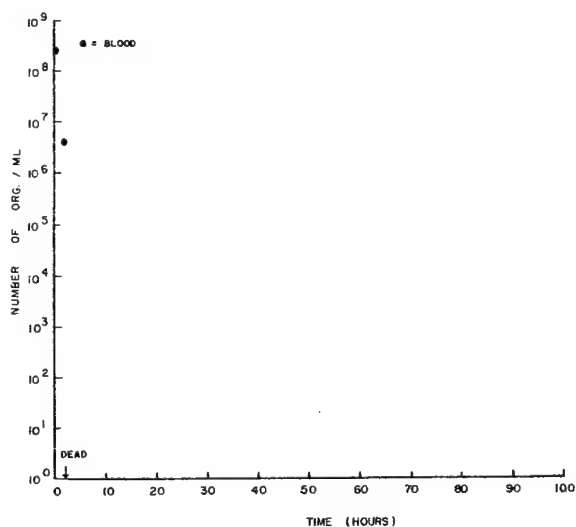
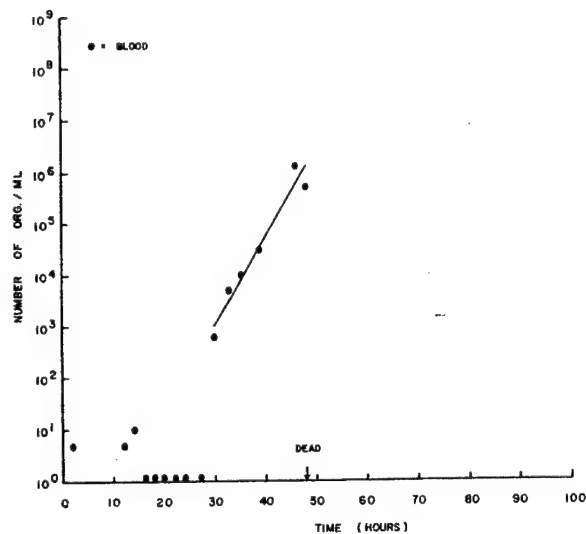Route   IV

Dose 1.14×10^{11} Spores
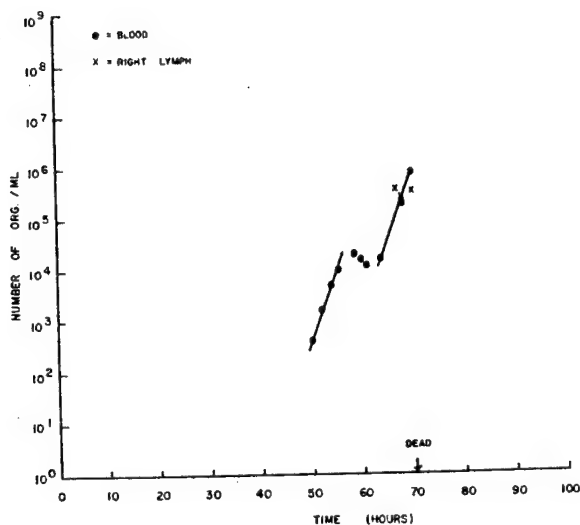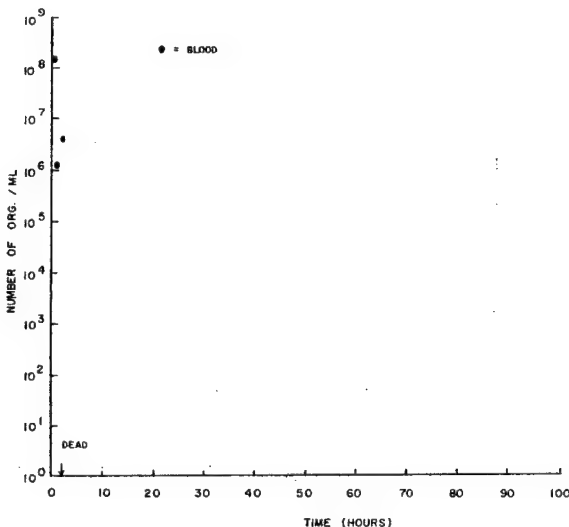
Route   IV

Dose 1.20×10^{11} Spores

Figure 11.   Bacilli per Milliliter of Blood or Lymph of the
Rhesus Monkey Challenged by Indicated Route
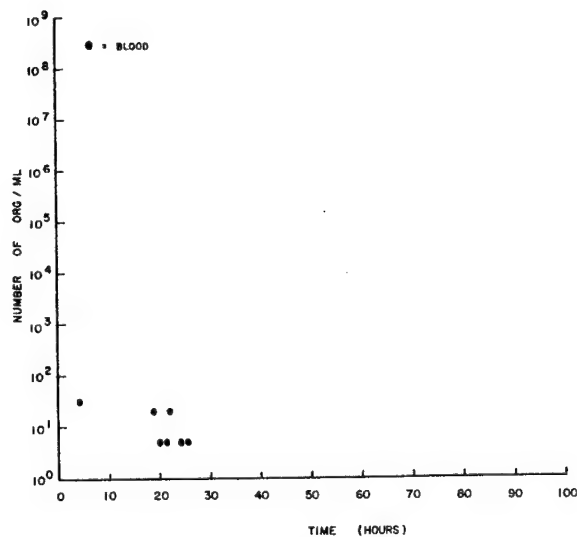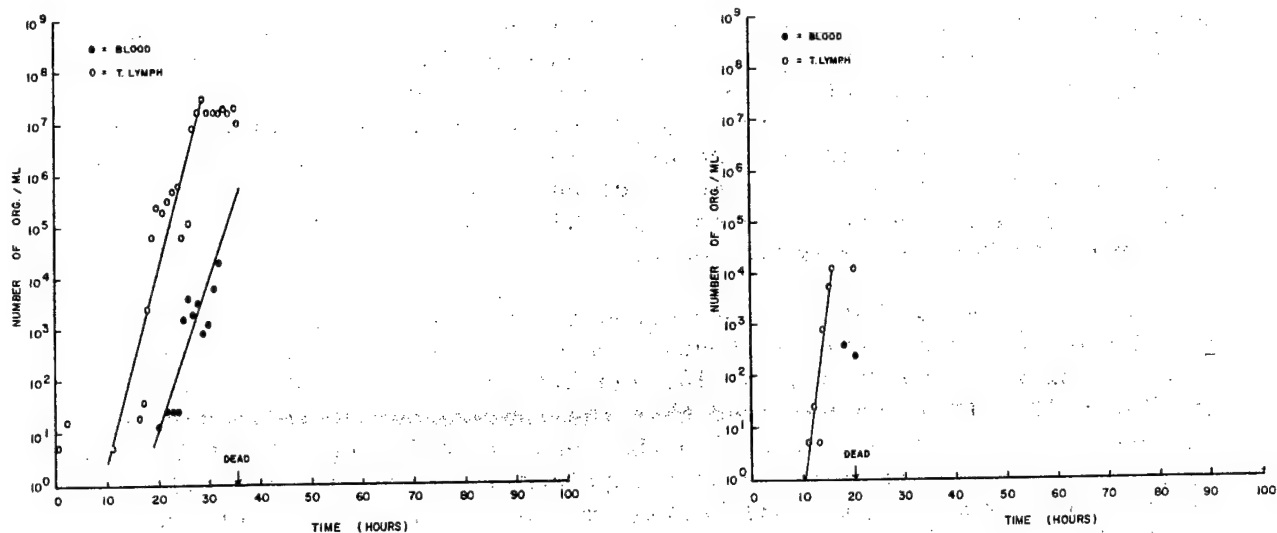and Dose of Virulent V1b Anthrax Spores.
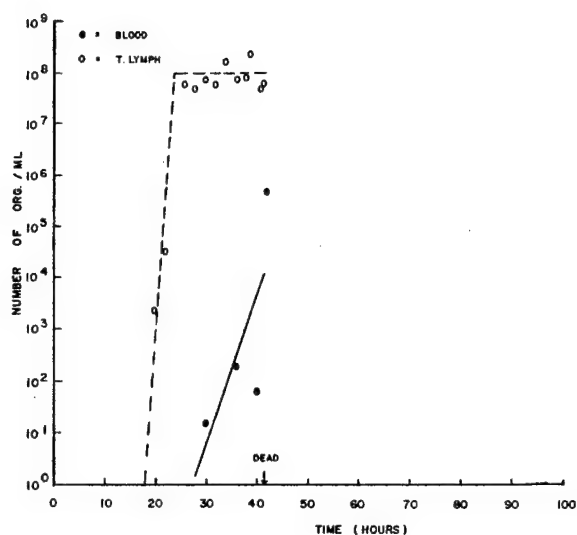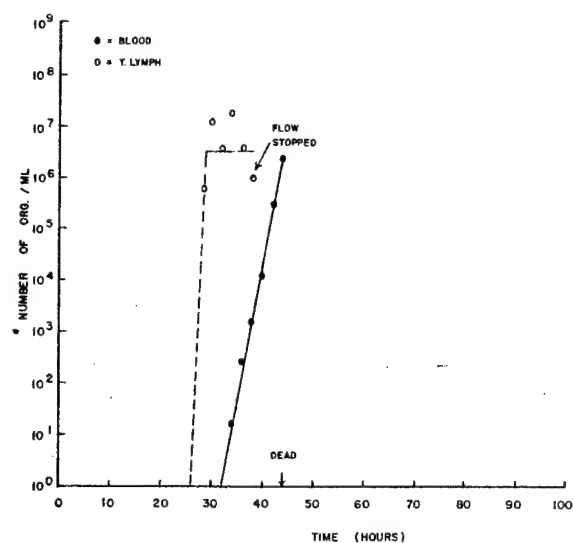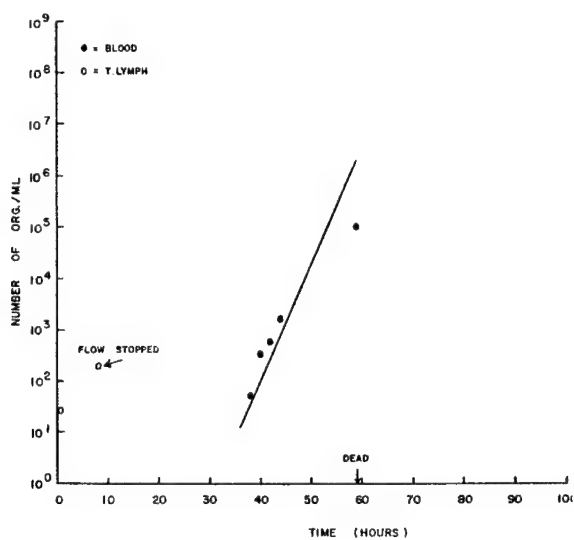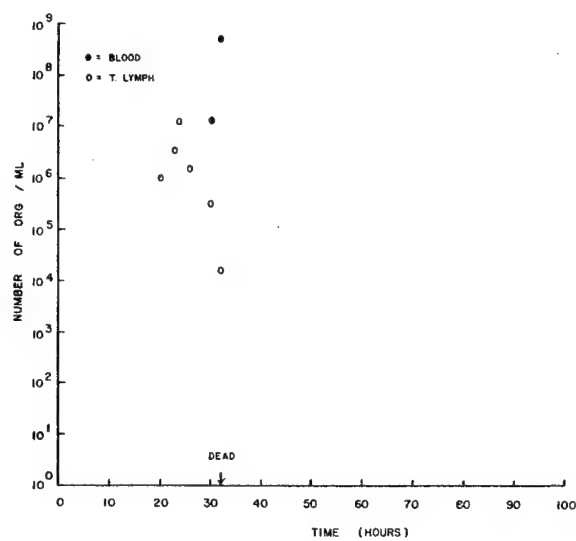
Figure 12. Bacilli per Milliliter of Blood or Lymph of the Rhesus Monkey Challenged by Indicated Route and Dose of Virulent V1b Anthrax Spores.

During the course of our work on pathogenesis in which the course of anthrax and the development of septicemia in the rhesus monkey was studied, the terminal blood of 15 rhesus monkeys was tested for toxin. In 12 of these terminal blood samples toxin was demonstrated by death of the rat. We have observed a relationship between terminal level of bacilli/milliliter of blood, time-to-death after challenge, and presence of toxin. When death occurs early after challenge, the terminal concentration is high and toxin is present; the converse is true when time-to-death is long. These observations probably account for absence of toxin in three of the monkeys reported above.

Evidence for increasing concentrations of toxin, to a maximum concentration at death, was obtained from two monkeys. The thoracic lymph duct and the jugular vein of the first monkey were cannulated. Twenty-four hours post-surgery, the monkey was inoculated intradermally with 10^5 virulent anthrax spores. The site at which the inoculum was placed drained to the popliteal lymph node. The animal died 41.5 hours after challenge. Lymph was collected regularly during that time, and the samples were kept separate so that changes in the lymph could be correlated with time. Changes in organisms and toxin levels are shown in Table III. The results of three assays of each sample are shown. For instance, the lymph collected from this monkey at 17 hours prior to death was found to contain $<0.2 \times 10^6$ organisms/milliliter; lymph collected three hours prior to death contained 88×10^6 organisms/milliliter. During this same period, the toxin titer increased from zero to a 1:5 dilution. When one milliliter of the three-hour lymph sample was injected intravenously into each of two rats, they died in an average time of 144 minutes, in contrast to the fact that those injected with lymph collected 13 hours before death survived. From this table it can be seen that the lymph collected immediately before death of the monkey was more antigenic and more lethal to rats than lymph collected several hours prior to death. This observed increase in toxin is statistically significant (P <0.001).

Sterile lymph collected before challenge was not toxic to rats. It was also proved that a combination of two parts of the toxic lymph and one part of anthrax antiserum* was nontoxic. The above tests demonstrate that death was caused by toxin produced by B. anthracis infection.

The second experiment was similar to the first, except the right lymph duct, which drains a major portion of the lungs, was used instead of the thoracic duct. Also, the monkey was challenged by the aerosol route with 3×10^5 anthrax spores. The data on this monkey, which died in 57 hours, are shown in Table IV. Since the lymph flow was very slow only a small amount could be collected over any time period. Thus the toxin test used only one rat per point and challenge was with only 0.5 milliliter of lymph. Therefore, the times-to-death on this table do not compare directly with those on the preceding table. The regression of time prior to death of the

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* Anthrax spore antiserum from hyperimmunized horse (DH-1-4A).

TABLE III. ANTHRAX ORGANISMS AND TOXIN IN LYMPH FROM MONKEY
CHALLENGED BY INTRADERMAL ROUTE

| Time Prior to Death, hours | Organisms in Lymph, 10^6/ml | Tests for Toxin | |
		Rat, min. to death	Precipitin, titer
17	<0.2	S[a]/	0
13	52	S[a]/	1:3
11	71	360	1:4
9	69	233	1:4+
7	168	143	1:5
5	85	126	[b]/
3	88	144	1:5
Death	69	76	[b]/

a. Survived.
b. Not enough lymph collected to run titer.

TABLE IV. ANTHRAX ORGANISMS AND TOXIN IN LYMPH FROM MONKEY
CHALLENGED BY AEROSOL ROUTE

Time Prior to Death, hours	Organisms in Lymph, 10^6/ml	Test for Toxin Rat, min. to death
8	15	Survived
7	18	240
5	19	237
3	15	180
Death	26	177

monkey on time-to-death of the rat has a slightly significant slope
(P <0.10). Although the level of significance is low (probably due to the
small number of rats), there is an increase in toxin concentration during
the period of observation. These observations parallel those made in the
previous experiment. The agar precipitation test was not performed on any
of these samples because of insufficient material.

The terminal blood of each of these two monkeys was assayed for toxin.
That of the first monkey was found to be negative, whereas the terminal
blood of the second monkey was toxic for rats. From Figure 13 it is seen
by the dotted line that the concentration of organisms in the lymph of the
first monkey built up rapidly to approximately 10^8 organisms per milliliter.
There was no such build-up however in the blood (solid line) of this animal.
It is shown in Figure 14 that the concentration of organisms in both the
blood and lymph of the second monkey increased to relatively high levels.
As with the first monkey, however, the build-up of organisms in the lymph
was greater than in the blood. It was also found that the terminal blood
of the second monkey was toxic for rats that died four hours after being
injected with 0.5 milliliter of the blood.

The fact that toxin was demonstrated in the terminal blood of the
aerosol-challenged monkey and not in the terminal blood of the intradermally
challenged monkey is not unexpected. It is believed that toxin was present
in the blood of the latter monkey, but below a demonstrable level. Since
toxin was demonstrated in the blood of the aerosolized monkey while the
lymph was being drained to the exterior, there must have been some other
route of entry of toxin into the blood. This presence of toxin in the blood
of the cannulated monkeys may be accounted for by: (a) a sufficient number
of organisms may enter the blood directly from the portals of entry and,
upon multiplication, produce a demonstrable level of toxin; (b) toxin may
diffuse directly through the wall of the lymphatic system because of increased
permeability; and (c) toxin may enter the blood after the establishment of
pathological secondary lympho-venous communications in the lymph nodes.
None of these possibilities were tested in our laboratory, but the work of
Malek et al[25] support the third possibility. They demonstrated in sheep
the development of secondary pathological lympho-venous communications in
the popliteal node draining a site of cutaneous anthrax. They further demon-
strated that these pathological communications developed during the inflam-
matory process of the node, through which organisms passed directly into the
blood. If, during an anthrax infection, organisms pass directly into the
blood from the lymph nodes, it is logical then to assume that toxin also
passes into the blood in the same manner. Therefore, if the above phenomenon
applies, the amount of toxin entering the blood directly is proportional to
the number of lymph nodes draining a particular site of challenge. In the
case of these monkeys, the number of lymph nodes draining the lungs of the
aerosolized monkey is far greater than the number of lymph nodes draining
the site of inoculation (below the popliteal node) in the intradermally
challenged monkey. Hence, it is a question of the number of lymph nodes
and the proportional development of secondary pathological lympho-venous
communications between the two lymph systems that should account for the
absence of toxin and low concentration of organisms in the intradermally
challenged monkey.
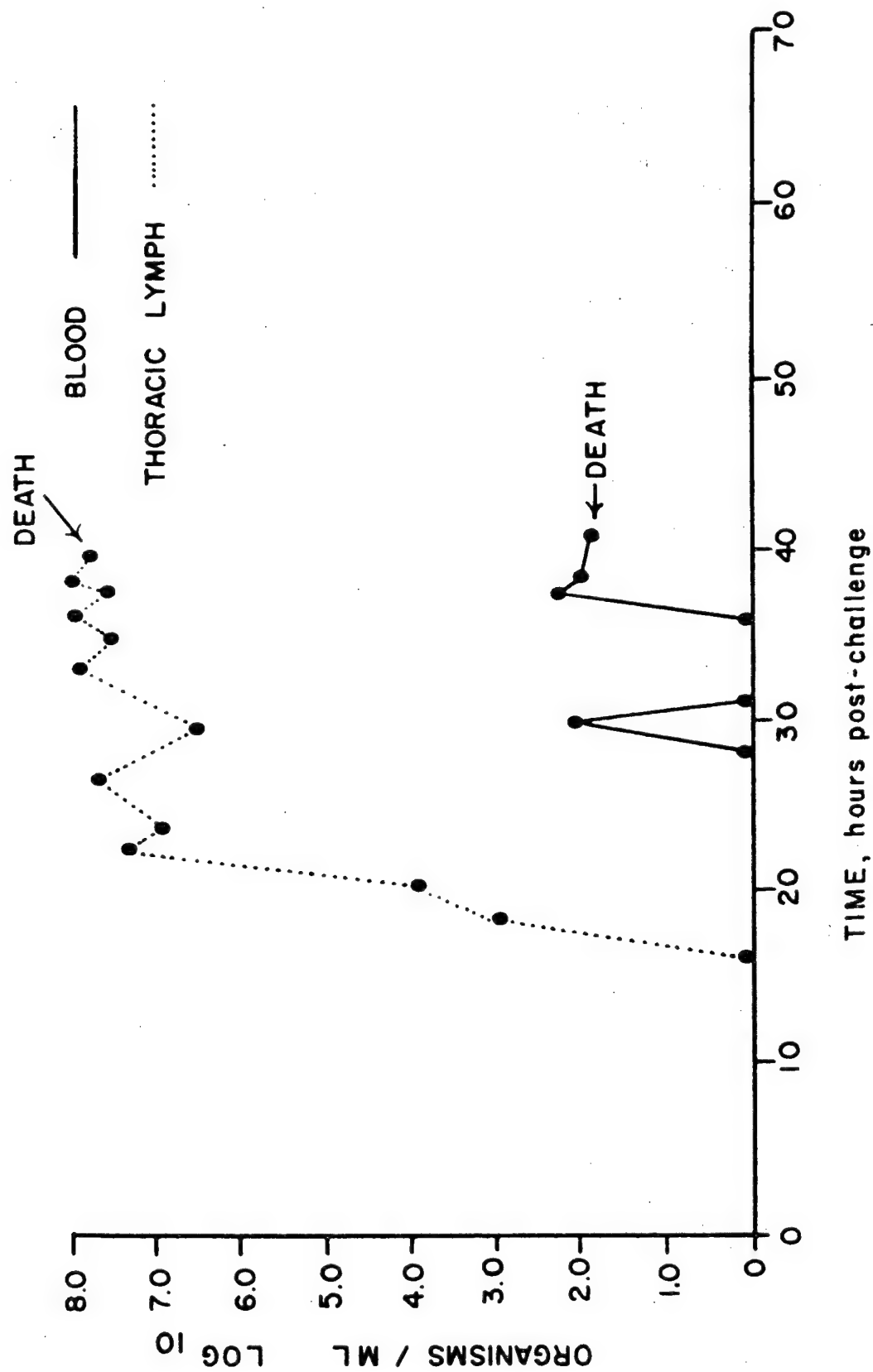
Figure 13.   Bacilli in the Blood and Thoracic Lymph of a Monkey Intradermally Challenged with Spores of B. anthracis.
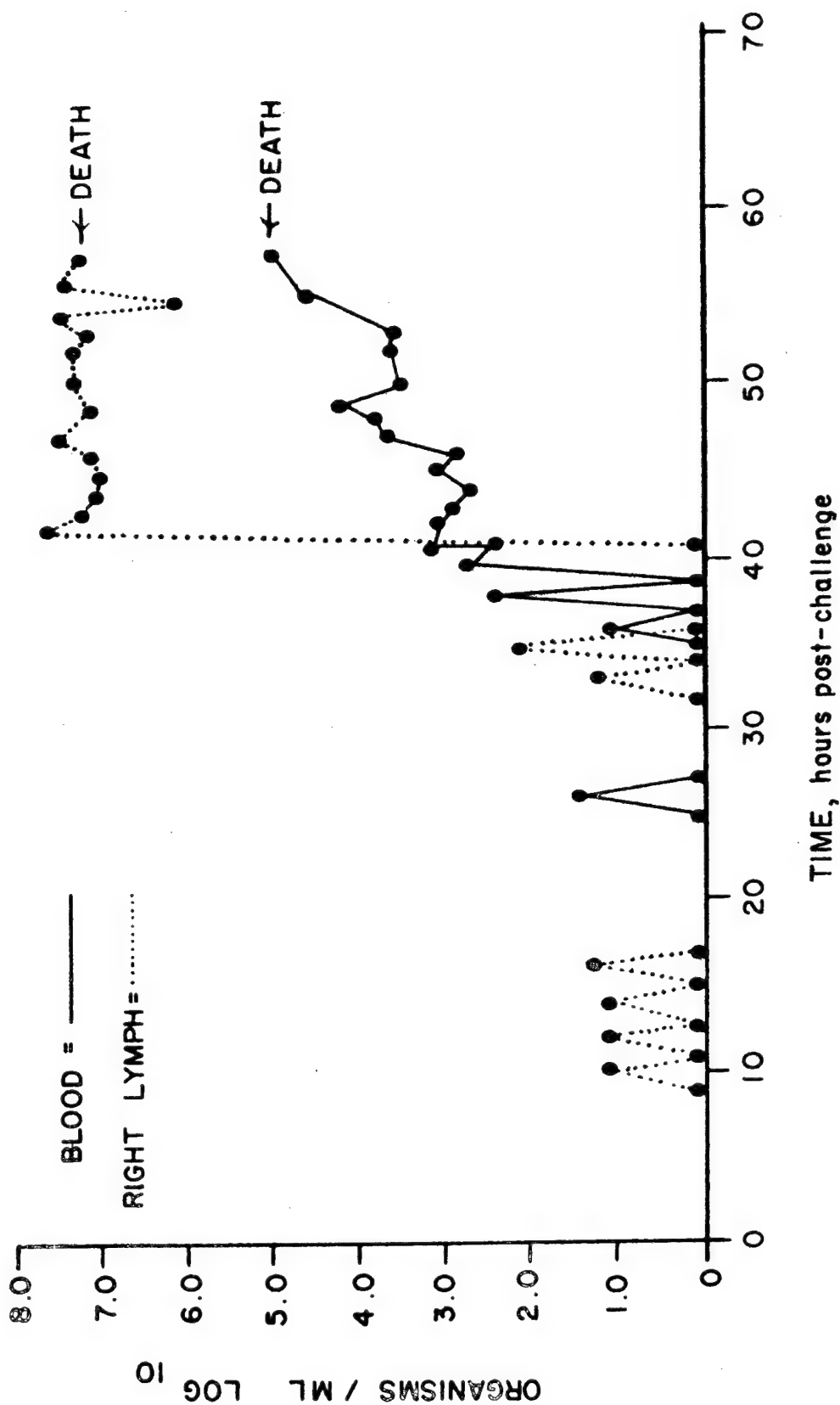
Figure 14. Bacilli in the Blood and Right Lymph of a Monkey Following Aerosol Challenge with Spores of B. anthracis.

These data show that toxicity is proportional to the concentration of organisms in the body fluids. Specifically, the lymph of both monkeys showed parallel increases of both organisms and toxicity. The blood of the first showed a negligible increase of organisms and no toxicity, whereas the blood of the second showed a substantial increase in organisms accompanied by a demonstrable increase of toxicity. This is the first demonstration of such a build-up of toxin in body fluids of monkeys and complements the British observation[3] on toxin in terminal blood of guinea pigs.

The next step was to show that anthrax toxin alone is sufficient to cause the death of these animals. Sterile toxin was produced _in vitro_ by the method reported by Beall _et al_[23] and of a potency such that one-milliliter doses killed rats in an average of 80 minutes. A dose of 120 milliliters of this toxin plus 250 milliliters more at 24 hours was administered intracardially to a rhesus monkey weighing 16 pounds. The monkey received the toxin with no apparent ill-effects. It died 45 hours post-challenge with terminal symptoms associated typically with toxic death in rats (i.e., partial to complete paralysis of upper extremities, extreme respiratory distress, and pulmonary edema).

In another trial, three eight-pound rhesus monkeys were challenged intravenously with 200, 250, and 400 milliliters, respectively, of sterile anthrax toxin produced _in vitro_. A fourth monkey received 250 milliliters of toxin, followed by three 50-milliliter injections of antiserum given zero, one-half, and 21 hours post-challenge. The animal receiving 200 milliliters survived; the animals challenged with 250 and 400 milliliters died in 60 and 33 hours, respectively. The animal receiving toxin plus anthrax antiserum survived. These data indicate: (a) a possible dose-response relationship between anthrax toxin and death of the rhesus monkey and (b) that toxin was neutralized by antiserum. The volume of liquid administered, although large, did not noticeably affect the monkey, and it is to be noted that the surviving animal that received antiserum also received the same amount of liquid as the monkey dying of toxin and, in addition, received 150 milliliters of antiserum.

The final evidence of toxemic death of rhesus monkeys was revealed when a dose-response curve for challenge doses of 10^5 through 10^{11} spores was run. The expected dose response was observed for the doses 10^5 through 10^{10} spores, with death occurring between 20 and 50 hours. Three monkeys given 10^{11} spores, however, died two hours after challenge with symptoms of toxemic death. Since 10^{11} particles could be toxic by themselves, the same inoculum of 10^{11} spores was reduced to 10^{10} viable spores by heat-shocking. On injection of this dose, the course of the disease corresponded to that for 10^{11} viable spores, showing that the rapid death was not due to an increased number of particles. We interpret these observations as showing that 10^{11} spores, on germination and outgrowth, produce enough toxin to kill a monkey. An entirely different course of the disease as evidenced by short time-to-death and only symptoms of toxemia indicate that these three monkeys died of an anthrax toxin.

In this work, it has been shown that: (a) anthrax toxin can be demonstrated in body fluids of the rhesus monkey at death; (b) anthrax toxin is present in increasing amounts in body fluids of the rhesus monkey dying of anthrax; (c) in-vitro-produced, sterile anthrax toxin is capable of causing the death of rhesus monkeys; and (d) anthrax antiserum protected a rhesus monkey against in vitro toxin produced by B. anthracis.

The importance of toxin in affecting the pathogenic course and, therefore, the treatment of the disease in man, remains to be determined. It seems probable that the effect of toxin in man is comparable with that in other animals and, if so, this additional knowledge of toxin has a significant implication on the pathogenesis and treatment of the disease. The possibility of a contrary view must be recognized, particularly in view of the observations of The U.S. Army Medical Unit at Fort Detrick, in the 1958 fatal anthrax case[26] who was hospitalized for over 110 hours before death. They said "...at no time during his illness did this man show any evidence of a systemic toxin effect." To the authors, the extrapolation from the Porton work is more convincing and the parallelism is remarkable. By this interpretation, a critical concentration of toxin was surpassed. Organisms were then largely removed from the body by antibiotic treatment, thereby removing the production of toxin. Death occurred from toxin at a time much later than if the septicemia had been allowed to progress uncontrolled.

Reinterpretation of the 1958 case was developed earlier[27] from the specific history of the case that is given in Table V. The zero hour of onset of disease is taken arbitrarily as 0900, 29 June, on the general information that the subject remarked that he did not feel well while he was preparing to attend church services. It is noted that the 1300-hour blood sample obtained on 30 June was negative by direct blood smear, negative by agar plate culture when the plate was examined after incubation for 20 hours, but positive with many colonies/cm^2 when re-examined at 44 hours. Anthrax, being a vigorous fermenter and grower, would have appeared as relatively large colonies if the agar had been seeded at the time the blood sample was added; therefore, it is concluded that the agar was seeded secondarily, i.e., from the fluid in the bottom of the flask, at 20 hours while inspecting it for primary growth. Since anthrax was present and was not impinged on the agar, only a few cells/sample were present. This number is taken conservatively as one cell/ten milliliters of blood, because ten milliliters was the sample size used. The contaminated sample taken three hours later does not contribute or detract from this deduction; consequently, it is deleted. The next significant observation was made at 0730 on 1 July (46-hour sample). The blood continued negative by direct blood smear, but was positive on agar smear when examined after 24 hours of incubation. It is known from our work with guinea pigs, rats, and mice that if 10,000 cells per milliliter of blood were present, that about three cells would be observed/100 microscope fields; consequently, the number of cells per milliliter of blood drawn at 0730 on 1 July contained less than 10,000 cells/milliliter. It has been assumed that 1000 cells/milliliter were present, and this number probably is nearly accurate, because later, careful examination of the entire stained slides showed two anthrax-like cells. Thus, the number increased

TABLE V. DATA AND CALCULATIONS BASED ON ONE SEPTICEMIC CASE OF ANTHRAX

Date	Hour	Activity	Results	Cumulative Hours	Org/ml	Total Number of Bacilli
6/29	0900	Onset of symptoms	-	0	-	-
6/30	1200	Admitted to hospital	-	27	-	-
6/30	1300	Blood culture	neg - 20 hr pos - 44 hr	28	0.1	550
		Blood smear	neg			
6/30	1600	Blood culture	Coli contam	*	*	*
7/ 1	0730	Blood culture	pos - 24 hr	46	1000	5.5×10^6
		Blood smear	neg		(<10,000)	
7/ 1	1600	Blood culture	pos - 24 hr	55		
7/ 1	1900	Rx	-	58	2.56×10^5	1.4×10^9
7/ 3	1330	Blood culture	neg	100	-	-
7/ 5	0100	Death	-	136	-	-
7/ 5	?	Tissue culture	pos	-	-	-

- No basis for estimating data.
* Data not pertinent.

from 0.1 cell/milliliter at 28 hours to 1000/milliliter at 46 hours. This is 13 doublings in 18 hours, giving a calculated doubling rate of 1.42 hours. Treatment was started at 1900 on 1 July, which is 12 more hours of uncontrolled growth, and at the calculated rate, this would result in nine more doublings, bringing the cell number to 2.56×10^5/milliliter of blood. A blood culture taken three hours before treatment started was positive; a culture taken 45 hours after initiation was negative. The lack of a smear on the last positive culture or of quantitative counts on any blood culture eliminates the possibility of determining the accuracy of the above deductions. It may be noted in passing that a quantitative value of the terminal number of bacilli/milliliter at death of man would be of extraordinary value if tests with man are ever planned. Such evidence may be available in case histories of woolsorters disease.

The number, 2.56×10^5 bacilli/milliliter, is believed to be a minimal figure because (a) it is not a terminal observation but rather a post-critical level, (b) tetracycline is a bacteriostatic-type antibiotic, and (c) fermentation, if not multiplication, would continue for some time after administration of the antibiotic. The positive tissue isolation 78 hours post-administration of antibiotic also supports the view that cell destruction is slow; therefore, fermentation and toxin production should continue for a significant period after antibiotic is administered. The statistic is given further credulity by its fit in the regression of body weight plotted against total number of organisms in the blood stream at death for all animals for which data are available (Figure 15). A blood volume of eight per cent of body weight was assumed in developing this regression. The data for mouse (25 grams), rat (240 grams), guinea pig (350 grams) and monkey (5250 grams) are from our own work; the chimpanzee (10,500 grams) are based on the publication of Albrink and Goodlow;[38] and man (80,000) are based on the calculation given above terminating in 2.56×10^5 cells/milliliter of blood, although as discussed above, this figure almost certainly is low. This statistic fits the regression remarkably well. It indicates that the assumptions made for the deduction with a human case fit remarkably well with experimentally determined information. It is one more bit of information indicating that extrapolation can be made from laboratory animals to man.

G. DEATH OF THE HOST: HEMATOLOGY, SYMPTOMATOLOGY, AND GROSS PATHOLOGY

1. Hematology

Hematological studies were carried out on several monkeys, but only two were followed at regular intervals throughout the course of the disease. Table VI shows the blood picture of a monkey challenged intraperitoneally with 1.1×10^6 spores. The differential count revealed a shift to the left (increased percentage of neutrophilic cells) after surgery, with no significant change in the total red and white cell count. There was, however, a slight variation in the size of the red cells. Post-challenge, the percentage of segmented cells increased slightly until the tenth hour when there was a
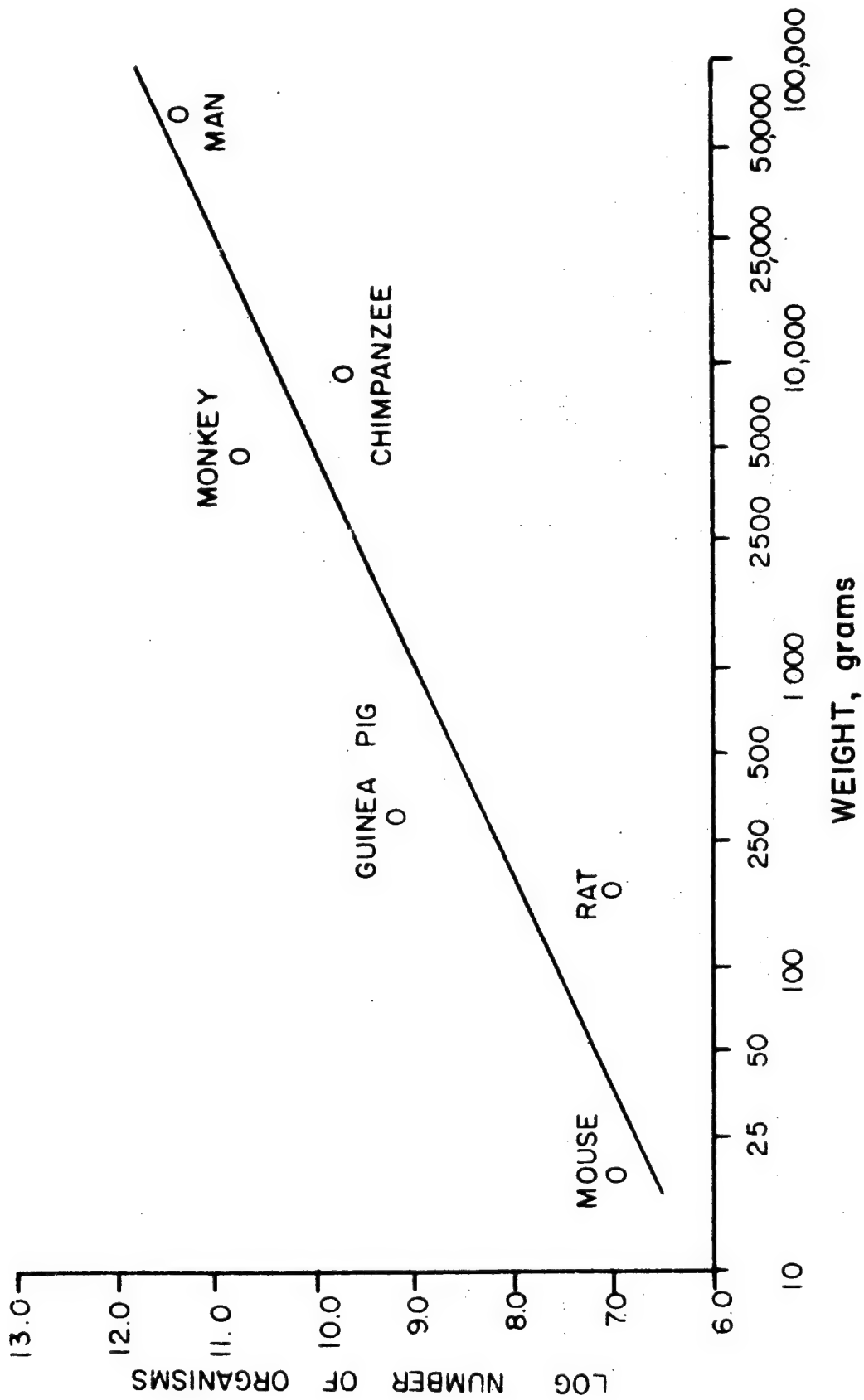
Figure 15. Regression of Total Number of Organisms in Blood to Body Weight of Animal.

TABLE VI. DESCRIPTION AND KINDS OF BLOOD CELLS OBSERVED IN MONKEY #13
BEFORE AND AFTER CHALLENGE INTRAPERITONEALLY

	Seg	Ban	Juv	Lym	Mon	Eos	Mylo	NRBC[a]	Description of RBC Variation	Total Count RBC	WBC	Hb
Before Surgery	36	1		57	1	3			Typical	3.5x10⁶	19,350	12.8
0 Hour	71	6	1	17	5				Anisocytosis, Slight	3.4x10⁶	17,750	9.4
1 Hour	74	2		22	2				Anisocytosis, Slight			
5 Hours	79	5		13	3				Anisocytosis and Poikilocytosis			
10 Hours	82	3		13	1	1			Anisocytosis and Poikilocytosis			
15 Hours	49	29	4	12	5		1		Anisocytosis and Poikilocytosis			
18 Hours	20	62	8	21	6	2	1		Same, More Marked			
20 Hours	9	32	4	49	6			6	Same, Very Marked	2.9x10⁶	4650	9.1

a. Nucleated Red Blood Cells.

decrease until death. On the other hand, the percentage of bands remained relatively constant until the fifteenth hour when there was a marked increase, with a drop off at death. A few juveniles appeared from the fifteenth hour until death. The description of the red blood cells revealed a variation in size and shape, with a very marked degree of variation at death with a decrease in the total red and white blood cell count. The hemoglobin showed no significant change post-challenge.

Table VII shows the blood picture of a monkey challenged via the aerosol route with 5.6 x 10^5 spores. This monkey differed from the first, in that no change was observed in the differential count before and after surgery, and there was no change in the size and shape of the red blood cells. There was an increase in the total white blood cells at death, as contrasted with a decrease in the intraperitoneally challenged monkey.

Table VIII presents the cellular changes in the lymph of the intraperitoneally challenged monkey, and Table IX shows the cellular changes in the lymph of the aerosol-challenged monkey for which the blood observations were given earlier. After surgery, the lymph of both monkeys showed a slight increase in the percentage of segments and no significant change in the lymphocytes. There was, however, an increase in the total white cell count. Post-challenge, the differential count revealed a slight increase in the percentage of segments in the intraperitoneally challenged monkey and a marked increase in segments in the aerosol-challenged monkey. The percentage of lymphocytes, in contrast, decreased in the aerosol-challenged monkey until death and remained fairly constant in the IP challenged monkey. The total white cell count in both monkeys varied slightly without trend throughout the course of the disease.

The hematological studies of these two monkeys did not reveal any findings not expected in a typical bacterial infection. These studies are not complete as more extensive and complete blood work is being pursued in an attempt to understand this disease better and to determine whether any observations may prove useful in the early diagnosis of anthrax.

2. Symptomatology

Our observations were made on rhesus monkeys receiving parenteral or aerosol doses high enough so that most animals died 48 to 96 hours post-challenge. A few animals lived as long as six days.

Although most of the 36 animals challenged had been operated on 24 or 48 hours earlier, in no case was there evidence of anthrax infection of the wound and, except for the first two animals challenged, all were free of streptococcus or other contaminants throughout the experiment.

No symptoms were observed until eight to ten hours before death. At this time, the monkeys' faces became flushed and a ring formed around their eyes. The ring darkened as the disease progressed. The animals remained alert until they reached a moribund state from three to four hours to a few minutes before death. Most animals ate readily until the moribund state was reached.

TABLE VII. DESCRIPTION AND KINDS OF BLOOD CELLS OBSERVED IN MONKEY #11
BEFORE AND AFTER CHALLENGE VIA AEROSOL

	Seg	Ban	Juv	Lym	Mon	Eos	Mylo	NRBC	Description of RBC Variation	Total Count RBC	WBC	Hb
Before Surgery	65			27	8				Typical	5.6×10^6	15,500	11.3
0 Hours	65	7		28	7				Typical	5.4×10^6	21,800	11.1
1 Hours	74			17	2			1	Typical			
4 Hours	37	4		54	5			1	Typical	4.4×10^6	15,400	11.1
9 Hours	46	4	1	44	6			3	Typical			
14 Hours	49	16	1	27	7			3	Typical			
18 Hours	48	25	1	18	8			1	Typical			
21 Hours	57	13		16	14				Typical			
24 Hours	54	7		23	16		1		Typical	3.8×10^6	22,100	14.9
25 Hours Term.	35	18	3	29	13			8	Typical			

TABLE VIII. DESCRIPTION AND KINDS OF LYMPH CELLS OBSERVED IN MONKEY #13 BEFORE AND AFTER CHALLENGE INTRAPERITONEALLY

	Seg	Ban	Juv	Lym	Mon	Eos	Total Count WBC	RBC/100 WBC	Bacilli Chains
Pre-Exposure	12			85		3	2100	20	None
0 Hour	10			87		3	6450		None
1 Hour	9	2		84	4	1	6200		None
5 Hours	10			88	1	1	10,200	80	None
10 Hours	14	2	1	81	2		9300	146	None
15 Hours	17	7	1	75			10,100	257	None
18 Hours	19	1		80	1		11,900	156	None
20 Hours	15	2		82	1		9600	52	10

TABLE IX. DESCRIPTION AND KINDS OF LYMPH CELLS OBSERVED IN MONKEY #11
BEFORE AND AFTER CHALLENGE VIA AEROSOL

	Seg	Ban	Juv	Lym	Mon	Eos	Total Count WBC	RBC/100 WBC	Bacilli Chains
Pre-Exposure	3			92	5		1600	None	None
0 Hours	6			90	3	1	5300	None	None
1 Hours	8			91		1	16,400	None	None
4 Hours	3			96		1	5200	None	None
9 Hours	10			84		6	9400	None	None
14 Hours	35	2		56		7	Clotted	None	None
18 Hours	65	2		30		3	4500	None	None
24 Hours							5750	None	None
25 Hours	48	2		42		8		None	None

3. Pathology

There were no massive gross lesions, such as those typical of anthrax in epidemics among domestic animals, i.e., massive hemorrhages with blood oozing from the body's natural openings. A uniform observation is that terminal blood is dark and "tarry," more or less hemolized, and non-clotting. In all cases, the heart and lungs showed petechia and eccymmosis in varying degree. The pulmonary lymph nodes were slightly enlarged and hemorrhagic in aerosol-challenged monkeys, but there was no gross change in these nodes when challenge was intravenous, intraperitoneal, or intradermal. The spleen varied from slightly enlarged to markedly contracted; the liver varied from normal to slightly enlarged. The intraperitoneally challenged cases produced from slight to extensive ascites. Hemorrhage and enlargement of the mesentery lymph nodes occurred in a few of the intraperitoneally challenged cases and were absent in all other cases. Other than specifically stated for the pulmonary and mesentery lymph nodes, there was no generalized enlargement of the lymph nodes. Only nodes that drained the route of entry were enlarged and hemorrhagic. Congestion of the brain was observed in most animals and a few showed massive hemorrhagic meningitis. In most respects, symptoms of the disease were very similar to those described for humans. There were no constant symptoms, including temperature change, that indicated early infection.

There are a number of reports on detailed pathology, the most recent of which may be taken as representative.[29] The uniformity of the results may be noted, also that little understanding of this disease has been gathered by the extensive histological studies. New concepts and approaches should be brought to bear on problems approached histologically and, at this time, the evaluation of the effect of toxin separated into its components on tissue damage appears promising.

H. STATISTICAL MODEL OF THE PATHOGENESIS OF ANTHRAX

In the previous section of this report, we have presented a great many data along with our interpretation of them. The regularities we observed in these data have stimulated us to develop a mathematical model of the pathogenesis of anthrax. The model parallels the hypothesis presented earlier, in that it describes the course of fatal anthrax. It enlarges upon the hypothesis, in that disease is described in terms of parameters that can be quantitated. Through the model we expect to quantitate enough host responses so that we may safely bridge the gap between experimental species and man.

It has been shown that with anthrax, as with other species, the concentration of organisms in the blood of the host falls off at a very rapid rate after challenge with a dose D. According to the working hypothesis, these organisms are sequestered in such organs as the liver, spleen, lymph nodes, etc. The effect of the factor responsible for this rapid clearance can be represented pictorially (Figure 16). We assume here that this factor acts to produce clearance at an exponential rate. This is in conformity with other biological systems.
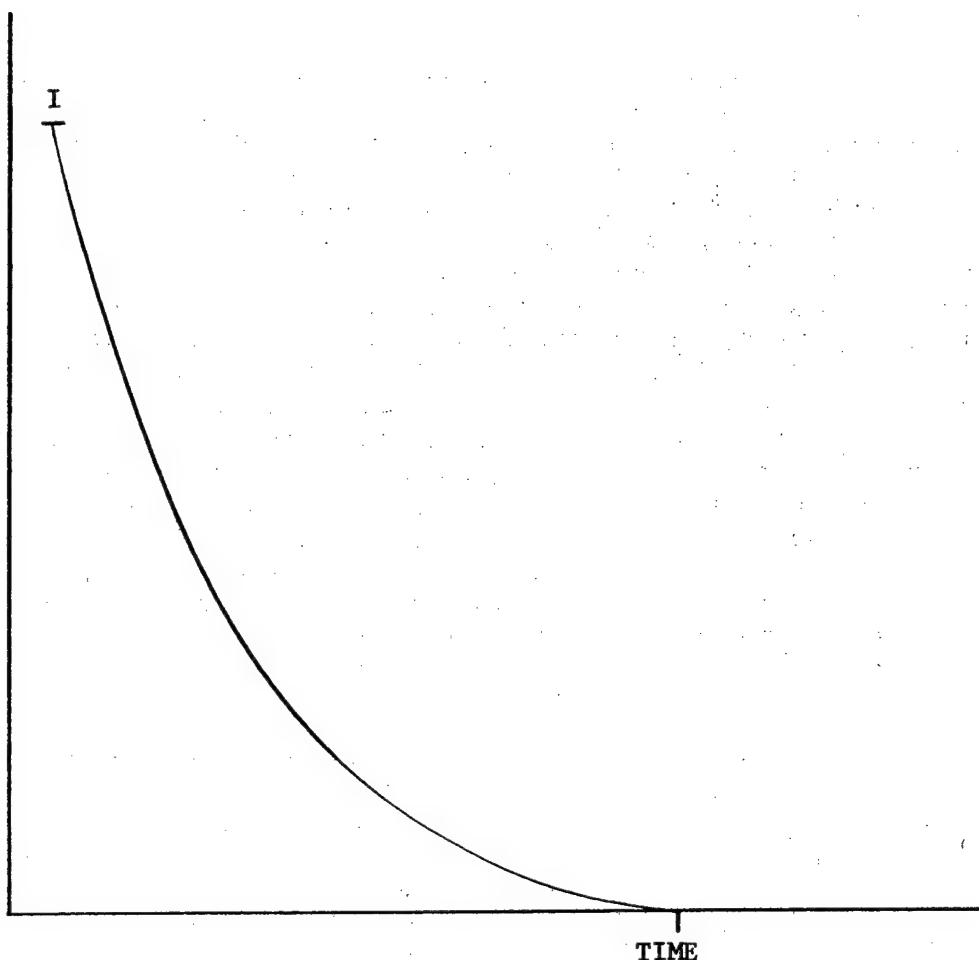
Figure 16. Anthrax Model 1.

Bennett and Beeson[30] have reported that a "remarkably stable" concentration of organisms is maintained by the net activity of the RES after this rapid clearance. We know very little about the mechanisms underlying the events that follow this initial clearance of organisms from the blood. Blooms of bacteremia may be due to intravascular multiplication or to a re-seeding of the blood from the organs most active in trapping the organisms initially. Regardless of the biological mechanisms involved, it is clear from our own work, as well as the work of others, that some factor must act to prevent the clearance from becoming complete. This factor must at least counteract any active resistance of the host and is represented in Figure 17 as the curved line labeled R1, which builds up from zero, crosses the line C representing the factor responsible for the clearance of the organisms,
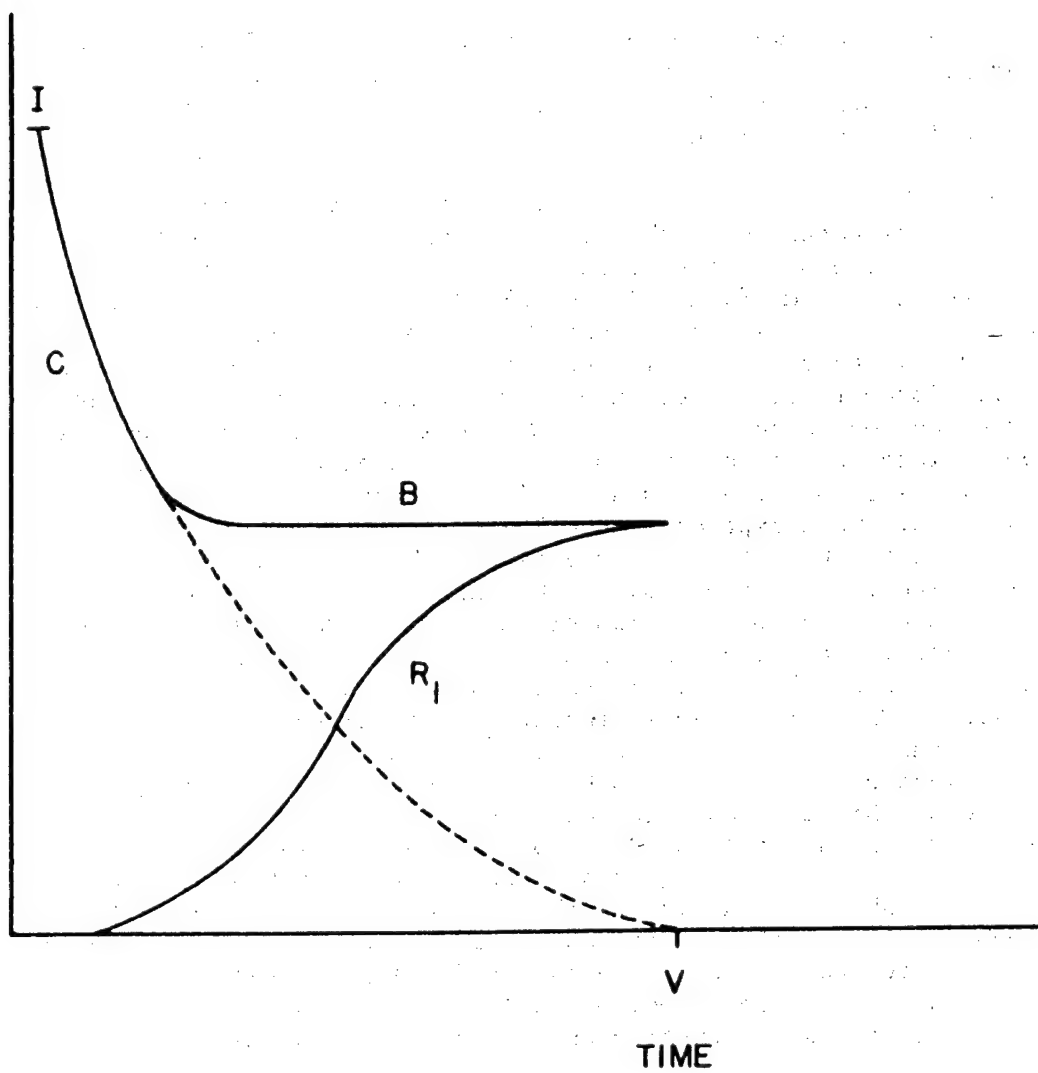
Figure 17.   Anthrax Model 2.

and approaches some maximum level designated on this figure as the line labeled B. Line B also represents the resultant or sum of the effects of these two factors C and Rl. It represents a close agreement with Bennett and Beeson's "remarkably constant" level.

During the stage of the infection, which is characterized by this constant level of bacteremia, the host through its resistance mechanisms will be contesting the organisms in an attempt to prevent a secondary septicemia. At some point in time (V in Figure 18), this interaction between the host and the organisms will reach a critical point at which the outcome of the infection will be decided. If the infection is put down, the host will recover. However, if the organisms are sufficiently virulent they will overcome the host's active resistance and a secondary septicemia will result. The work presented in the previous sections show that in fatal anthrax, septicemia develops at an exponential rate that is constant for any given host species. This exponential build-up is represented by line S. The resistance with which the host fights off the organisms is represented as line R2. The resultant or sum of the effects of the two factors R2 and S is represented by a continuation of the line B from time V on. Obviously when the active resistance R2 is completely gone, the lines B and S will coincide. The resultant of all these factors is shown in Figure 19 as a heavy line. This line then represents the model that describes the general course of the disease. The model can be quantitated in terms of concentration of organisms in the host's body. At the present time, quantitation must be done through studies of organisms in the blood, because measurements of organisms in the whole body is not at present feasible with large animals. However, work is progressing toward obtaining whole-body determinations, using small laboratory animals. The development of the model to this stage has enabled us to design experiments for the sole purpose of generating data from which to refine our ideas and develop a mathematical expression of them. At the present time, our only means of quantitating anthrax in any host species is by counting the number of organisms in his blood.

Since immediately after challenge, organisms could be most easily found when the challenge route was IV, and when it was desirable to test the validity of the model, we gathered data on the concentrations of organisms in the blood of monkeys that had been challenged intravenously. The concentration of organisms in a series of blood samples from a monkey challenged intravenously with 10 billion anthrax spores is shown in Figure 20. From this it is seen that the general shape of the theoretical model is consistent with reality.

The data from several nonimmunized monkeys challenged at several dose levels were examined and, from these, the following postulates were derived for the development of a model.

(a) Postulate 1 - The growth of the organisms in the host is influenced by active and passive resistance of the host.
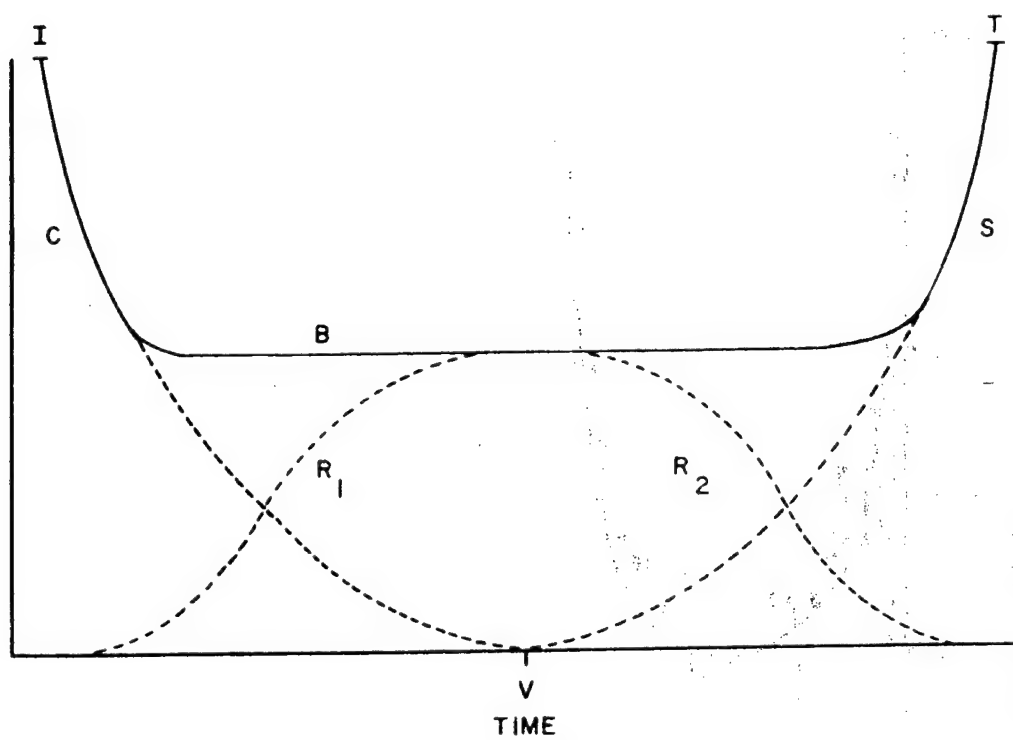
Figure 18.   Anthrax Model 4.
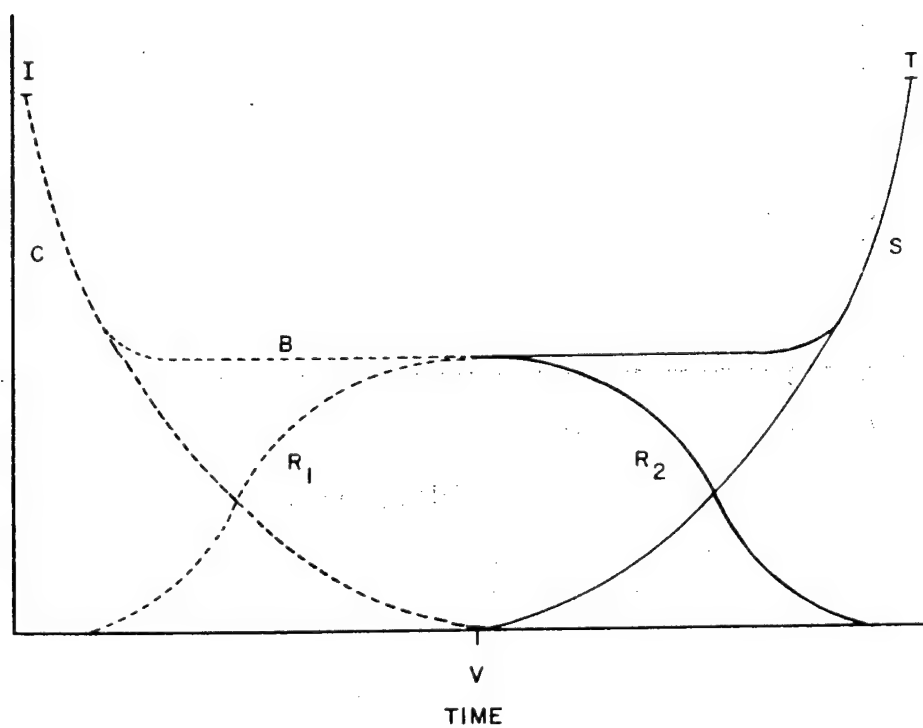
Figure 19.   Anthrax Model 3.
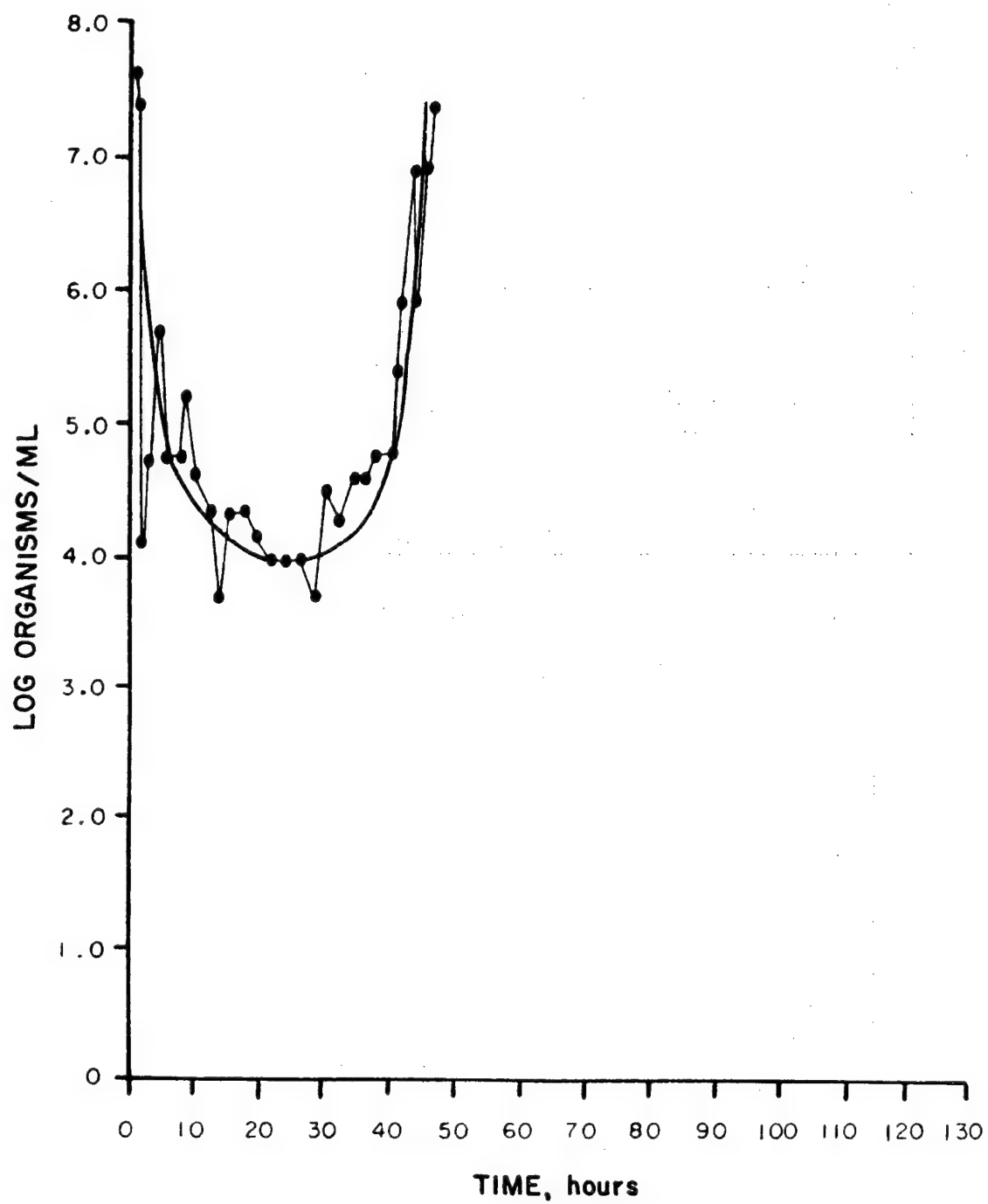
Figure 20.   Concentration of Organisms in the Blood of
Guinea Pigs from IV Challenge Until Death.

Active resistance is defined as that resistance that results from stimulation of the host by the presence and activity of the organisms in the host's body, e.g., phagocytosis and immune reactions.

Passive resistance is defined as any resistance due to conditions of the host's body that are independent of any direct responses of the host to the organisms, e.g., pH, absence or presence of amino acids, and body temperatures and so forth.

(b) Postulate 2 - The expression of active resistance is a function of time that approaches zero prior to the death of any host with a fatal case of anthrax.

(c) Postulate 3 - Passive resistance is a function of concentration of organisms and time.

(d) Postulate 4 - The net growth rate of the organisms starts at a certain point that may be either positive or negative and increases asymptotically to a maximum.

If we represent the natural doubling rate of the organisms by p and the natural death rate by q, then under natural conditions the net growth of the organisms can be described by the differential equation

$$\frac{dy}{dt} = (p - q) \, y \tag{1}$$

where y is the concentration of organisms at some instant of time t. The assumption that p and q are functions of time is consistent with other theories, e.g., the theory of mass action.

Postulates 2 and 3 demand other functions to represent both active and passive resistance of the host. The active resistance must be a function of time, which becomes zero for high values of t. According to the biological model, it should be symmetrical to a vertical axis and have a maximum. If we designate this function as g(t) then

$$g(t) = Ae^{-mt^2} \quad Ae^{-mt^2} \tag{2}$$

will satisfy all the requirements.

Passive resistance is, according to the postulates, a function of concentration and time that does not vanish. We can designate this function as h(t,y) and let

$$h(t,y) = (o + nt)y \tag{3}$$

According to the postulate, the net growth rate is the combined result of these three forces. Thus we have in a host

$$\frac{dy}{dt} = (p-q + o + nt)y + Ae^{-mt^2} \tag{4}$$

Now $(p-q + o + nt)$ is a function of t, which we may define as the function $f(t)$. This function can plausibly be represented by the equation

$$f(t) = a + 2bt \tag{5}$$

which is the familiar equation for a straight line. With this, Equation (4) becomes

$$\frac{dy}{dt} = a + 2bt + Ae^{-mt^2} \tag{6}$$

that is, a first order differential equation the solution of which

$$y = De^{-b(t-t_o)^2} + Te^{-m(t - t_d)^2} \tag{7}$$

The biological interpretations of the parameters in Equation (7) are

D = initial level

T = terminal concentration

b = growth rate when active resistance is absent

m = duration of active resistance

A = maximum level of active resistance that will be reached at time V

t_o = time of challenge

t_d = time of death

y = concentration of organisms at time t

The results of our application of the model to actual data that we obtained from several animals are shown by the following series of graphs (Figures 21, 22, and 23), in which the broken lines connect actual data points from three of the monkeys and the solid lines were calculated from the data with the model.
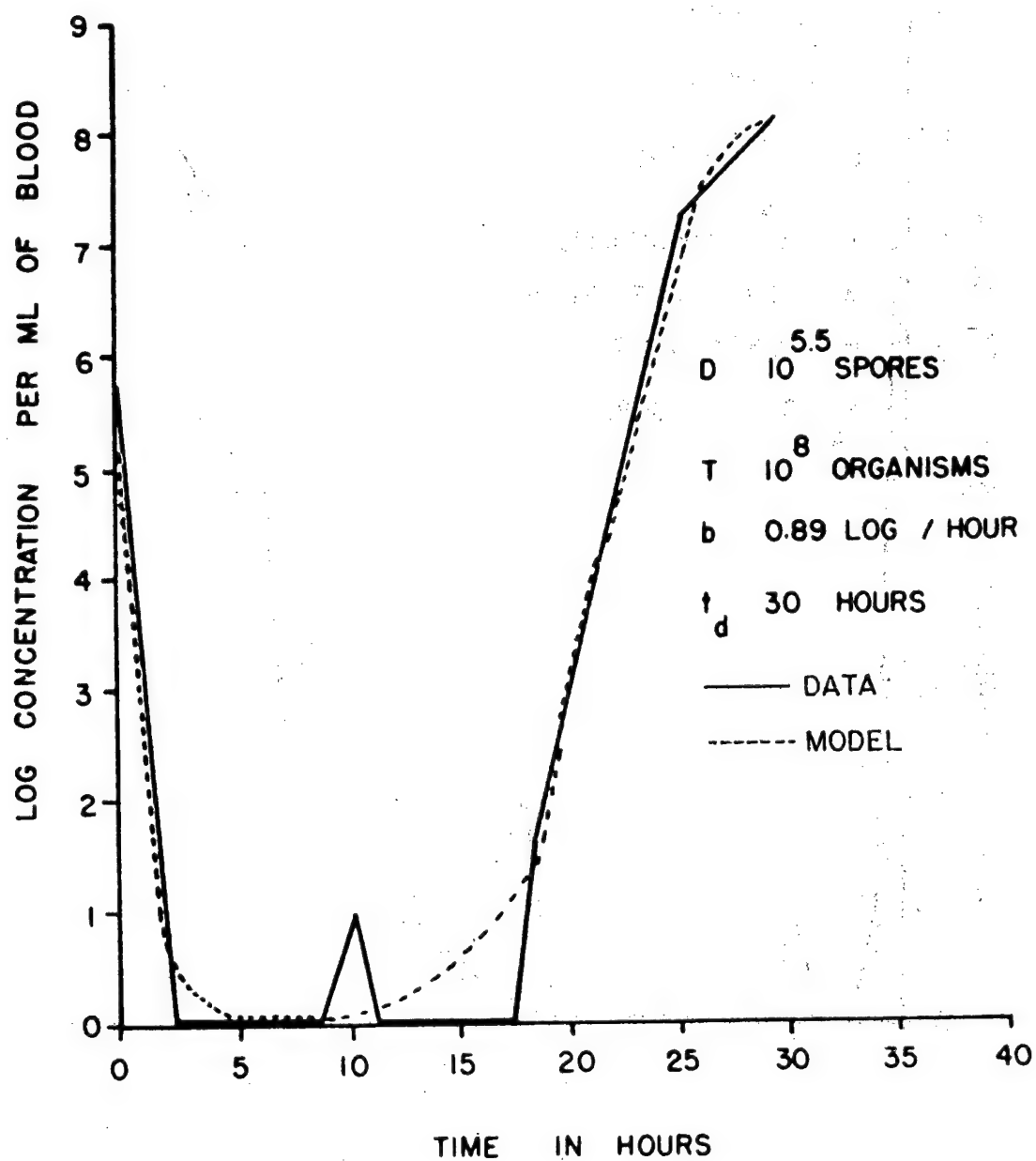
Figure 21. Relationship of Model to Actual Data Obtained
from Monkey Following Challenge.
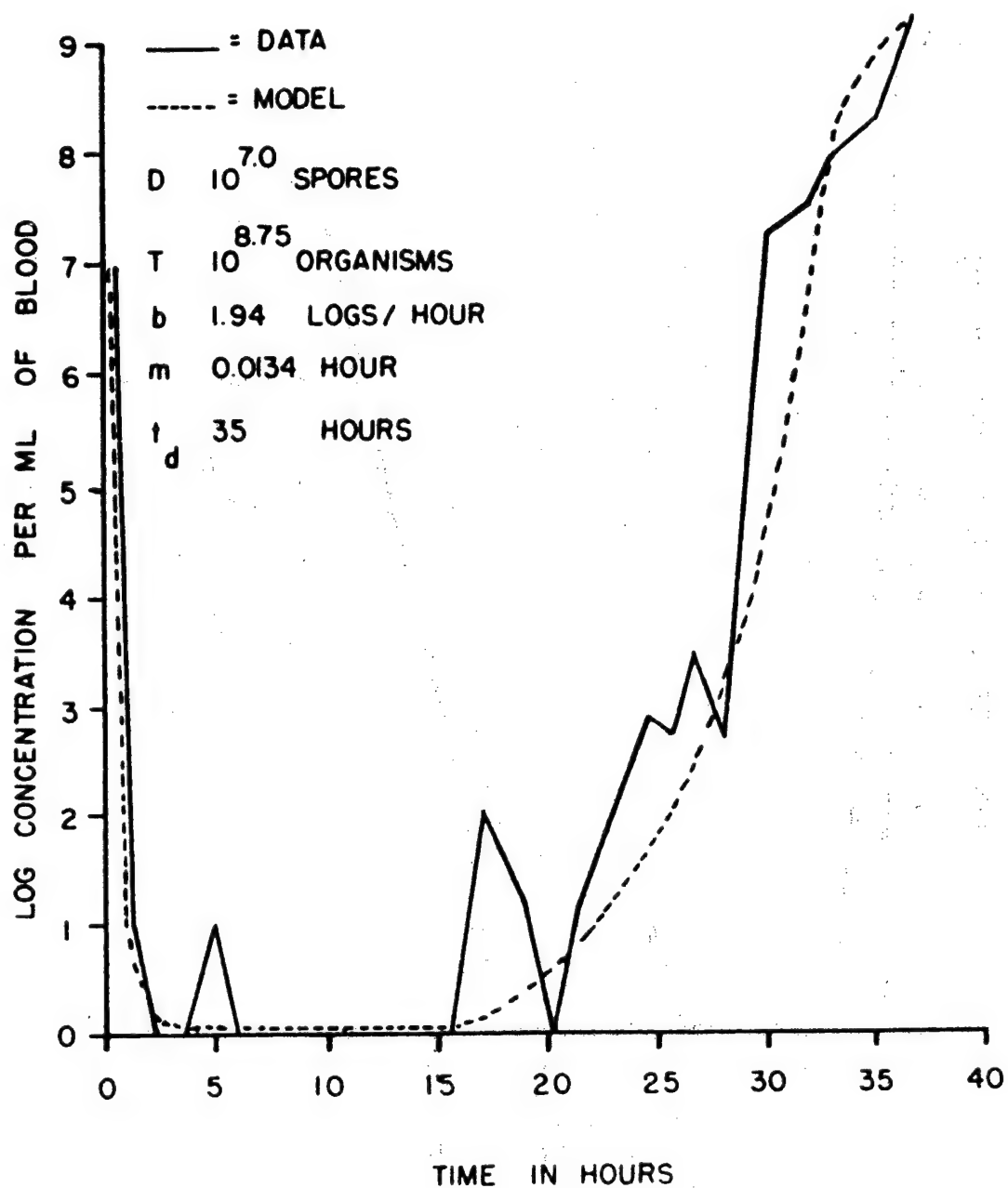
Figure 22. Relationship of Model to Actual Data Obtained from Monkey Following Challenge.
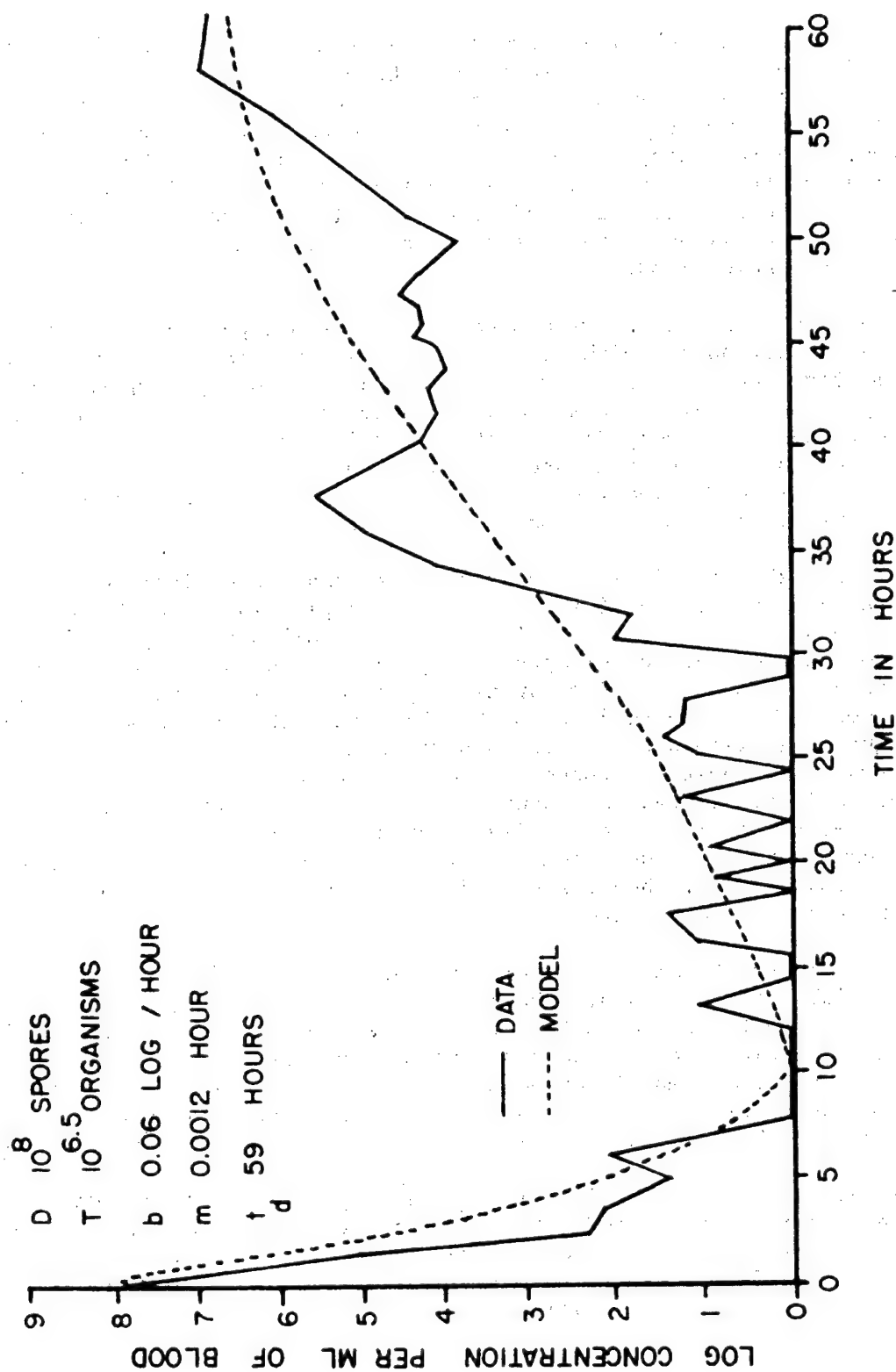
Figure 23. Relationship of Model to Actual Data Obtained
from Monkey Following Challenge.

In Figure 21 data obtained from a monkey that was challenged intravenously with $10^{5.5}$ anthrax spores are presented. Blood samples taken one and two hours post-challenge show the rapid disappearance of the organisms from the blood. No organisms were found between the second and eighteenth hour after challenge, with the exception of the 11-hour sample. At 18 hours, apparently the active resistance had disappeared, whereupon the organisms began increasing at an exponential rate of about 0.89 log per hour. The monkey died 30 hours after challenge with a terminal concentration of 10^8 organisms per milliliter of whole blood.

These data in Figure 22 were obtained from a monkey that had been challenged intravenously with 10^7 spores. Again the rapid disappearance of organisms from the blood is shown by the first two hourly samples. Following this, organisms appeared sporadically at 6 hours and 18 hours. The final septicemic stage, however, did not start until 21 hours after challenge. At this time the active resistance was overcome and the organisms increased at an average rate of 1.94 logs per hour until the host died with $10^{8.75}$ organisms per milliliter of blood at 35 hours.

In Figure 23, data from a monkey that was challenged intravenously with 10^8 spores are shown. The first two hourly samples show the characteristically rapid decrease in concentration of organisms. Following samples, however, they show a slower rate of disappearance, which becomes complete only after seven hours. This condition of apparently no organisms continued until the twelfth hour. From the twelfth to the thirtieth, the counts we obtained were higher than zero and tend to support Bennett and Beeson's observation of a "remarkably constant" concentration during the presepticemic stage. The septicemic stage began at about the thirtieth hour and continued until the thirty-sixth hour, at which time it appears that the active resistance again became evident. The second septicemic stage started at 49 hours and continued to 59 hours, when the host died with a terminal concentration of $10^{6.5}$ organisms per milliliter.

Up to this point, all laboratory studies and predictions have been conducted on laboratory animals. The last—and most crucial phase of this program—will be the extrapolation from animal to man. This will require the early detection and efficacious treatment of the disease. Then, if transformation of the model can be made to aerosol challenge, learning to use this model successfully along with adequate treatment of laboratory animals should instill the necessary confidence to proceed with challenge in man.

Presently, work on the model is progressing on two fronts. One is the actual programming of the model to quantitate the parameters associated with the intravenous route of challenge. The other is the development of the model from a different set of underlying factors whose parameters are more adequately expressed biologically.

IV. SUMMARY OF WORK DONE SINCE THE REPORT DATE, AND GENERAL PLANS FOR IMMEDIATE FUTURE WORK

A. SEPARATION, PURIFICATION, AND CONCENTRATION OF ANTHRAX TOXIN, AND DETERMINATION OF THE EFFECT OF THE PURIFIED ANTIGENS ON PATHOGENESIS AND IMMUNITY*

A program was initiated to separate, purify, and identify the components of anthrax toxin. The three known components of the toxin have previously[22,31] been labeled as (a) factor I, or edema factor, which in combination with protective antigen is not lethal to rats but gives a positive skin test in guinea pigs; (b) factor II, or protective antigen, which is not lethal to rats and gives a negative skin test in guinea pigs; and (c) factor III, or rat lethal factor, which in combination with protective antigen is lethal to rats and gives a negative skin test in guinea pigs.

The in vitro toxin was produced by the method used by Beall et al,[23] then lyophilized after stabilization with ten per cent normal horse serum. Storage was at -25°C.

Ten different ion-exchange cellulose samples were checked for their ability to retain the lethal factor in toxin. One of these, #71 DEAE type 20, gave a good flow and retained or neutralized the lethal factor.

At this time, the edema factor can be separated from toxin by filtering toxin through an ultrafine sintered glass filter. The filter factor is retained on the filter and can be eluted from the filter with carbonate buffer. If the modified toxin that passed through the sintered glass filter is now passed through the DEAE column, the rat lethal factor is inactivated or removed while the protective antigen passes through the column. To date, elution of the rat lethal factor from the column has not been achieved.

Preliminary studies indicate that some of the toxin components can be separated by electrophoresis. A larger electrophoresis tube has been fabricated and will be used in an attempt to further purify the three known components.

The effect of pH through the range of toxin was examined and no immediate effect on lethality for rats was shown; however, after 24 hours of storage at 4°C, the samples that were acid had lost their lethality in rats. Therefore, the pH of the toxin should be kept above 7.0.

The effect of temperature on toxin was also studied. The toxin lost its lethality for rats after three hours at room temperature or after 30 days at 4°C. It stores well at -25°C.

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* Bill G. Mahlandt and Charles Wigington.

After separation and standardization of the three components of toxin, all 16 factorial combinations of these antigens and live vaccine will be tested to determine their effect on pathogenesis and immunization. It is particularly desired to develop a specific toxin-neutralizing antiserum for possible use in treatment of generalized anthrax; it is also possible that a better method of immunization will be developed.

B. PHYSIOLOGY AND MEDICAL NEUTRALIZATION OF TOXIN*

In animals dying of anthrax toxin there is massive pulmonary edema, involvement of the central nervous system, and as reported by some workers— a classical case of cardiovascular collapse. In spite of the above syndromes, the electrocardiogram of monkeys remains normal throughout the course of the disease.[22] In monkeys, the central nervous system involvement appears to be of two stages: first, a spastic paralysis of the forearms is observed followed by a flaccid-type paralysis. From these observations we can hypothesize that, due to decreased cholinesterase, the acetylcholine activity effect on muscle fibers results in a spastic paralysis; then overcompensation of cholinesterase results in flaccid paralysis. However, the flaccid paralysis may be due to other factors. The white rat shows, besides a massive pulmonary edema, a stimulation (running and jumping) followed by extreme weakness just before death. The weakness observed in rat and the flaccid paralysis of the monkey may be in involvement of the central nervous system or anoxia. The rabbit shows paralysis with partial or complete recovery before death from toxin.

If treatment is started late in the course of acute anthrax, the infection can be arrested—yet the animal dies. Death in these cases is due to toxin being produced by the organisms (or possibly a secondary toxic substance whose production and release by the body is initiated by the anthrax toxin). Neutralization of the toxin or its effects, therefore, is the key to treatment of advanced septicemic anthrax.

In order to advance on the problem of neutralization and treatment of advanced septicemic anthrax toxin, it is desirable to obtain physiological data on the effects of anthrax toxin. We proposed to concentrate our efforts on determining the pathogenesis of toxin and to neutralize or medically overcome its effect upon the body in order to prevent toxemic death due to anthrax. We will obtain data on the action of selected drugs expected to potentiate or retard the action of the toxin on the body. Other procedures will be to observe the effects of toxin upon blood pressures throughout the body, brain waves, spinal cord, direct or indirect effect upon lung tissue, and various other physiological processes.

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* Lt. Jerry S. Walker and Lt. D. Fitzpatrick.

C. TISSUE CULTURE ASSAY PROCEDURE FOR EARLY DETECTION OF ANTHRAX IN A TEST HOST*

Experiments to date have been designed with the purpose of determining whether or not in-vitro-grown tissue isolates can be utilized as an assay system for the rapid detection of anthrax in test hosts. Preliminary studies indicated that these tests were sensitive, rapid, reproducible, and clinically economical and feasible.

A major portion of the work has been directed toward the development of a reproducible assay procedure. During this phase of the program in-vitro-produced anthrax toxin was used. The tests indicated that when in-vitro-grown anthrax toxin was overlaid on a tissue culture cell assay sheet of approximately 5×10^5 to 6×10^6 cells, no cytopathological effects (CPE) could be noted visually. When the concentration of cells challenged was reduced to 2×10^5 cells, a CPE could be elicited with either a 4X-, 2X-, or 1X-standardized toxin concentrate. Further reduction of the total tissue culture cell number enables detection of lower toxin concentrates, in addition to the concomitant rapidity of visual detection (2X, 18 minutes; 1X, 20 minutes; 0.5X, 32 to 35 minutes).

The single test to date for early detection of anthrax toxin in a test host was run on a surgically cannulated rhesus monkey challenged by the ID route with 1×10^5 Vlb spores. The monkey died of anthrax 40 hours after challenge. The first lymph sample, assayed in Fischer 344 albino rats by a one-milliliter injection into the ventral vein of the penis, in which toxin could be detected, was a lymph sample collected 12.5 hours prior to death (27.5 hours post-challenge). The time-to-death of the two rats assayed by this method was 113 and 117 minutes. Lymph collected from 18 hours prior to death (22 hours post-challenge), until death, and assayed in tissue cultures, proved to be toxic to the cells, eliciting a CPE in less than 40 minutes. Post-operative, prechallenged, autologous lymph, used as a control, had no effect upon the culture even after eight hours of exposure.

Tests to date indicate that the tissue culture cell assay procedure is more rapid and sensitive than the rat assay and shows promise as a device for early detection of the disease, a possibility that will be explored fully. Tissue culture studies also furnish opportunities for determining the effect of toxin on cellular physiology.

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* Sp5 Arnold Ross.

D. HEMATOLOGY AND BLOOD CHEMISTRY AND EARLY DETECTION OF ANTHRAX AS SHOWN
    BY CHOLINESTERASE INHIBITION*

Because the problem of early detection of anthrax is an important part
of the anthrax picture as a whole, and because very little work has been
done in this area, this task was undertaken, employing standard hemotological
methods, such as:

(a) Total leucocyte count

(b) Differential leucocyte count

(c) Total red cell count

(d) Hemoglobin

(e) Fragility of red cells

(f) Sedimentation rate

(g) Clotting time

The blood picture has been determined, following anthrax toxin adminis-
tration to the Fischer white rat, using these tests. A test for determining
cholinesterase activity in monkey blood following parenteral challenge with
spores has also been initiated and is now in progress.

Several of the methods employed have demonstrated that there are both
quantitative and qualitative alterations in the blood constituents. The
total white cell count shows an increase beginning at three to four hours
after injection and continuing through the seventh hour. This increase is,
on the average, on the order of 4000 to 5000 cells/cm^2 and is twofold in
some cases. This is obviously due to a marked increase in the number of
immature granulocytic cells released into the circulating blood as demon-
strated by differential white cell counts. These cells are primarily
juveniles and band cells.

The red cell count and hemoglobin content, although quite variable
following exposure, appears to follow no definite pattern.

Fragility of the red cells also apparently varies without pattern, but
shows a fairly consistent decreased resistance to graded dilutions of
hypotonic saline when the dose of toxin is in the sublethal range. This
agrees with reports from the literature.

Changes in the sedimentation rate are again difficult to interpret due
to inconsistency of results. On the whole, however, the trend is toward
increased sedimentation rate after injection.

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* Lt. David F. Fitzpatrick.

Clotting time is consistently increased, as determined by Sabraze's capillary tube method, from about two minutes to four to five minutes after five hours.

Cholinesterase activity determinations have been made, using both monkeys that had been cannulated prior to challenge and monkeys that had not received the venous cannulation but were challenged with the live agent. Cholinesterase activity was followed by the Bromothymol blue screening test adopted from the Limperos and Rauta method.[32] It has been shown that cholinesterase activity is completely inhibited about 18 to 24 hours post-challenge, which was at least 18 hours before death. However, marked inhibition also occurs following venous cannulation, thus the value of this test is somewhat vague at this time. These observations were repeated using the quantitative method of Fleischer et al[33] to measure cholinesterase activity in whole blood. Inhibition in both the monkey and rabbit was demonstrated.

It is quite evident that many of the constituents of the blood vary to the point of being erratic following toxin administration, and this pattern will also probably be observed following live-agent challenge. Although clear-cut patterns can be drawn in some instances, such as the invariable increased proliferation of immature white cells of the granulocytic type and the inhibition of cholinesterase activity as measured by the above test, these phenomena are also known to occur under conditions other than anthrax infection, which would involve severe stress of a nonspecific nature.

Additional studies on the total blood picture following toxin administration and/or bacterial infection will be continued. Changes in cellular components will be followed along the lines already initiated and, in addition, analysis of the chemical components of the blood, such as cholesterol, bilirubin, nitrogen, calcium, iron, etc., which can be achieved by use of ultra-micro-chemical analysis techniques.

The significance of cholinesterase inhibition, the possible use of this observation as a means of early detection of anthrax, and use of anticholinesterases in treatment to neutralize the effect of infection or of toxin per se will be studied.

E. GROWTH CURVES OF BACILLI IN THE WHOLE BODY AND DIFFERENT ORGANS FROM DOSAGE UNTIL DEATH*

The course of B. anthracis infection from exposure to terminal septicemia as reflected in the blood of several species of hosts and the lymph system of the monkey has been well established. However, a knowledge of the growth of the organisms in the whole body, especially in the vital organs during the entire course of the disease, is necessary for full understanding of pathogenesis. Determining the growth curve in the entire body

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and of the principal organs during the entire course of the disease may
make possible early diagnosis of anthrax by the employment of biopsy tech-
niques and will furnish information applicable to treatment.

In current experimentation, the mouse is infected by an intraperitoneal
injection of germinated spores of B. anthracis, sacrificed by means of CO_2
gas, skinned, then macerated by a Servall Omni-Mixer. When spores were
germinated in amino acids, it was impossible to obtain accountability of the
organisms injected, and this was true even when the germinated spores were
added to the fluid in which the mouse was blended. This problem was solved
by the addition of a minute amount of protein to the germination fluid.
Using spores germinated in this fortified amino acid germination fluid, it
is possible to achieve 99 per cent germination and to recover immediately
after inoculation over 95 per cent of the organisms with which the animal
was inoculated.

In preliminary experiments, the regression curve in the mouse receiving
an intraperitoneal injection of 1.5 x 10^7 germinated spores showed that a
95 per cent destruction of the organisms occurred during the first five hours
of infection. It is planned to develop regression curves on mice inoculated
with three levels of spores. Ratios of bacilli concentration in the liver,
spleen, kidney, and blood to the total bacilli population in the body at
various times during the course of infection from dosage until death will
be determined. The liver will be sampled because it is an organ that can
be biopsied. Acquisition of a Hamilton Beach heavy duty meat grinder makes
possible the extension of these studies to include other animals, such as
the rat, hamster, guinea pig, and the monkey. The phagocytic index also
will be calculated to determine whether the index remains constant or
changes with the course of disease. This work will be extended to furnish
information on treatment, in that the generation and death curve from dosing
to cure will be determined when antibiotics, antisera, or other treatment
is initiated to interrupt the growth curve. The same curves will be deter-
mined in host's immunization to different levels, so that passive and active
immunity may be contrasted. When the dynamics of this desease are under-
stood, then interruption of the course of disease, i.e., treatment, may be
initiated as a science rather than being conducted as an art.

F. GENETIC STUDIES WITH BACILLUS ANTHRACIS*

Genetic studies in the pathogenic species B. anthracis are designed
with several purposes in mind. The most obvious is to develop strains with
increased virulence. Understanding the hereditable traits that are respon-
sible for virulence and the mechanics behind their inheritance is perhaps
more important. For this purpose, gene transfer systems have been pursued
and both a transformation and a transduction system have been demonstrated.

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* Ira C. Felkner, A. J. Rosenwald.

A transformation system has been developed, which involved (a) securing mutant strains, (b) developing a method to extract DNA from lysozyme-insensitive <u>Bacillus</u> species, and (c) working out the details of a procedure to make cells competent.

1. Mutants Secured

Streptomycin-resistant and streptomycin-dependent mutants have been secured for genetic analysis, as well as phage-resistant mutants. The streptomycin-dependent strains may be useful for immunization, as well as for challenge, where precise control of the infection is desired. Other strains may have biological significance, because the antibiotic-resistant forms would complicate therapy and the phage-resistant strains would complicate diagnosis.

2. DNA Isolation

Deoxyribonucleic acid (DNA) can be isolated from lysozyme-insensitive <u>Bacillus</u> species, which include <u>B</u>. <u>anthracis</u> and certain strains of <u>B</u>. <u>cereus</u> and <u>B</u>. <u>licheniformis</u>. The procedure[34,35] employs washing cells first with ethylenediamine-tetracetate followed by treatment with 0.15 M trichloroacetate (pH 8), after which cells can be lysed with sodium lauryl sulfate and the DNA extracted with 90 cer cent phenol and further purified. The purified DNA of <u>B</u>. <u>anthracis</u> was found to have a Tm (temperature at which 50 per cent of the strands of the double helix separate) very close to that of many <u>B</u>. <u>cereus</u> strains.[35]

3. Achieving Competence of <u>B</u>. <u>anthracis</u> Cells

Cells are first grown in Brain Heart Infusion (BHI) broth overnight with shaking at 37°C, after which they are inoculated into Spizian's Minimal medium supplemented with yeast extract and casein hydrolysate and incubated with shaking at 37°C for four hours. They are then inoculated into minimal medium supplemented with casein hydrolysate + 1-tryptophan and incubated statically for two hours at 30°C. Cells are then mixed in minimal medium with DNA isolated from a strain having at least one trait distinguishable from the recipient population and incubated for one hour at 37°C. The transformed cells and controls are allowed to phenotypically express the newly introduced characteristic for which transformants are then selected.

Transformation has been successful for the locus controlling high-level streptomycin resistance in <u>B</u>. <u>anthracis</u>, and the frequency of transformation is between 0.1 and 0.2 per cent, which is at least as high as the frequency of transformation for this same marker in <u>B</u>. <u>subtilis</u>. Another trait has, in addition, been "transformed." This involves the introduction of DNA from a culture lysogenized with a temperate phage (PBA30) to a competent nonlysogenized culture. In this system, however, ultraviolet induction is necessary for expression of lysogenic centers in the newly "transformed" cells. The frequency of transformation here is, however, lower and is approximately 10^{-5}.

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A second type of gene transfer system that has qualities different from that of the transduction system of Stamatin[36] is in the preliminary state of development. The background of this system is briefly this: the V1b strain of B. anthracis appears to be lysogenized by at least two and possibly more bacteriophages. Treatment with a combination of oleic and oxalic acids in the medium appears to render the bacterium sensitive to its own phage because induction with ultraviolet light on a "noncured" strain causes the lysate to spot mildly on the "cured" cultures. However, clear-cut plaques have not been demonstrated. If, however, a streptomycin-resistant mutant is exposed to ultraviolet and its resultant lysate added to a "cured" strain, the gene controlling streptomycin resistance is transduced at a frequency between 10^{-5} and 10^{-6}. However, no single plaques have been demonstrated. The explanation to this phenomena may be that the "cured" strain is not completely free from its lysogenizing phage and that the phage induced from the streptomycin-resistant strain is very similar or perhaps the same with some modification. When the cured strain is exposed to this induced phage, it may inject its DNA in along with genetic material from its host bacterium. Being unable to multiply because of protection afforded by the phage still harbored in the "cured" bacterium, the introduced bacterial information is copied and expressed in the progeny.

Genetic studies in B. anthracis, such as the ones described here, have much promise in helping us to understand the pathogenicity of this strain. These studies can be expanded into studying the factors that are involved in virulence, two of which are known to be toxin production and capsule formation. Other factors that could be studied are the inheritance of sporogenesis, for which Stamatin[36] has already described a transducing system, and the effect of the lysogenic state on the cell. In addition, hybridization experiments involving closely related species may be possible under the proper conditions. Thus it can be readily seen that genetic studies on this organism could lead to quite fruitful results that have both immediate and future application.

G. REVISION AND DEVELOPMENT OF MODEL OF PATHOGENESIS OF ANTHRAX*

Advances have been made in the development of the mathematical model of fatal anthrax. The original model described in this report was derived through the mathematical description of postulated biological phenomena. The resulting model is a six-parameter equation consisting of the sum of two exponentials. Estimation of parameter values for any set of data from such a mathematical expression presents difficulties in several areas. One difficulty is the choice of one of several available computational procedures, no one of which is satisfactory from all points of view and all of which are computationally complex. This model has the further disadvantage that the biological interpretation of some of the parameters is obscure or impossible. Thus, further work on the model has been directed toward the development of a mathematical expression from which (a) the parameters will be easily estimable and (b) the parameters will have obvious and useful biological interpretation.

* B. W. Haines and PFC Dale Sloop.

At the present state in its development, the new model meets the second of these two criteria, that is, there are obvious biological interpretations for the parameters. Computational difficulties have not been overcome and use of an analog computer, in conjunction with the digital computer to simplify and speed this task, is being investigated.

At present this line of investigation seems to promise great advances. If parameters from a series of test animals can be estimated, parameters characteristic of the host species likely will be found. Other parameters may be characteristic of the route of challenge, the strain of organism, or some other controllable condition. If so, in a test case, the estimation of the remaining variable parameters should be a relatively uncomplicated procedure that can be performed very shortly after detection of the disease. It will then be possible to predict the course and final outcome of the disease, as well as describe it with biologically meaningful parameters.

H. TREATMENT OF GENERALIZED ANTHRAX*

There is little question that treatment of anthrax early enough in the course of disease will result in cure; however, when initiation of treatment is delayed until generalized anthrax is diagnosed as a bacteremia, recent history indicates that the bacteremia may be cured but the host dies. It seems obvious that either methods of earlier detection must be developed or, alternately, methods to treat late in the septicemic stage are necessary. In view of these conclusions we view treatment to mean the development of methods that will allow cure of the host at the latest possible stage in the course of the disease. Progress toward early diagnosis is reported earlier and is (a) inhibition of cholinesterase and (b) toxicity to tissue culture cell sheets. Initial experiments have been completed to determine which of the antibiotics are of value in treatment of established, generalized anthrax. Treatment work to date has been with mice. When given in a single injection, penicillin can cure infection later post-challenge than can streptomycin, aureomycin, terramycin, or chloramphenicol. Penicillin, streptomycin, aureomycin, and terramycin are about of equal value if treatment is begun early and continued through at least four days. Chloramphenicol is without value under the condition of our tests in treating this disease if treatment is initiated four hours post-challenge or later. Nonimmunized mice given protective antigen at the same time as they were challenged parenterally die from a significantly lower dose than the dose that killed mice not given protective antigen. Work with antibiotics will be extended to determine optimum schedules and combinations. The value of specific antisera alone and in combination with antibiotics will be determined. In addition, other treatments to extend the stage at which cure can be effected will be considered. These will include use of sera, blood extenders and fluids, control of the environment as regards temperature and oxygen, heart stimulants, drug treatment to control edema, anoxia and other symptoms of secondary shock, and any other drugs, materials, or procedures that may prove helpful. The control of toxemia seems essential. We may note that each of the other phases of work bears on treatment furnishing basic information to allow a scientific approach to the problem.

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* F. Klein, Lt. Jerry Walker.

V. DISCUSSION

It is realized that man will be challenged with live anthrax spores only after it is possible for laboratory animals (particularly the monkey and chimpanzee) to be challenged and routinely cured after establishment of generalized anthrax to a stage so that death would certainly have occurred if treatment were not initiated. Treatment, then, would seem to be the effort to emphasize. However, if treatment is viewed as interruption of the progressive course of disease interaction, then an understanding in both qualitative and quantitative terms of the normal course of disease and the variability expected in any response is necessary, otherwise only dogmatic, arbitrary treatment efforts may be attempted. Our appraisal of the literature on anthrax early in this program was that surprisingly little was known about the qualitative aspects of this disease and virtually nothing was known about the quantitative aspects. For example, there was no knowledge of early detection and virtually none of late detection, because systemic anthrax is not usually diagnosed before death. With the advent of antibiotics, all previous knowledge and practice on treatment of generalized anthrax was abandoned, and, since antibiotics were introduced, no comprehensive work has been done to experimentally determine the optimum antibiotic treatments. The presence and importance of toxin is often questioned, and treatment is not directed against toxin. All this is contrary to the exquisitely beautiful demonstration of toxin in guinea pigs by the Porton group[7] and later work on in-vitro-produced toxin. Differences in the progression of disease in different hosts, or differences attributable to infection by strains of different virulence or infection by different routes of challenge, had been emphasized without any apparent effort to find and quantitate the basic dynamic pattern of anthrax infection and disease. With this over-all appraisal of the literature, we concluded that only a broad approach, considering the fundamentals of anthrax infection, could possibly furnish the proper basis for treatment of generalized, septicemic anthrax. Such an approach has been pursued for two years, and one may reasonably ask what has been achieved toward obtaining the dose for man.

It is of value to simply present and actively consider concepts that lead to the challenge of man, thereby obtaining a defendable estimate of the dose required to establish respiratory anthrax. As long as the possibility of deliberate challenge is not mentioned or, mentioned and immediately disclaimed as murder, progress toward such an objective must be slow. We have repeatedly presented the concept that, after research that can now be firmly visualized, man can be safely challenged with anthrax. We note as a corollary statement that, until this feat is done, research on strains of increased virulence, DNA hybridization of species, and like expenditure of energy is of no biological warfare importance, because quantitation in man is essential to evaluate these efforts. We believe there has been some erosion of the dogmatic, irrational rejection of this concept. There is greater recognition that the requirements for deliberate challenge are different and less

exacting than for identification and control of a case of woolsorters disease. Once challenge has been made, there is no longer a question of identifying the causal organism, and a nonspecific disease indicator as, for example, cholinesterase inhibition, may be adequate for indicating the establishment of disease. We sense, too, that there is growing recognition that because a problem is difficult and the objective dangerous, if essential to national defense, then progress toward this objective can and must be expected.

Our work has shown the orderly sequence of events in the establishment of generalized anthrax from dose until death. Actually, as we pointed out earlier[10] the sequence of events is virtually identical or parallel with those observed in other diseases that have been investigated extensively as, for example, mouse ectromelia.[37] Some of the irregularities attributed to this disease are, in part, simply due to using the customary dosage in which only 50 per cent, more or less, of the challenged population become infected. The fact that infection is not visualized as a dynamic interaction between pathogen and host, in which successive steps will occur if the quantitation of the variables in the preceding step attain the necessary threshold but will not occur if this quantitative level is not reached, also contributes to the view that this disease is unpredictable. Interactions, then, must be investigated, as well as simple variables, such as dose, virulence, incubation period, and treatment. Thus a broad approach to this problem is mandatory. Without orderly progression, a model of infection dynamics would be impossible. Our problem in developing the model presented in this report has been in finding simple variables that can be evaluated and worked into a model, not in finding an ordered series of events.

The possibilities of using an indirect method to estimate the dose required to infect man via the aerosol route appeals to all because, presumably, the risk would be reduced. However, the degree to which risk is reduced must be compensated for by inability to defend the statistical estimation of dose. In view of the inability to defend previous arbitrarily arrived-at dose estimates, repeating this cycle once again does not seem constructive. Our quantitation of the septicemia occurring in immunized and nonimmunized guinea pigs and rats indicate that, because the terminal number of bacilli is lower in the immune host, an immunized host should not be subject to challenge. When an immunized host is used, the time between detection of the disease in the septicemic stage and a fatal toxemia is significantly reduced, compared with the course of disease in a nonimmunized host. We question whether a strain of low virulence should be used for challenge. This conclusion is made because of the higher dose required to establish disease, without other known benefits accruing as once established, the low virulent strain (30R) has the same doubling rate and terminal level in the blood as does the virulent strain (V1b). Since the terminal level remains the same, we believe that each strain, regardless of virulence, produces approximately the same quantity of toxin to cause death of the host.

This is another indication that death occurs once a given level of toxin is reached in the body. We re-evaluate the data on the Willard case[26] and make a convincing case for toxemic death of man. In spite of the specific statement that no evidence of toxemia was observed during 120 hours of observation, it is difficult not to recognize a possible close parallel between (a) the Willard case and the Boyles case in which a septicemia was controlled for more than 48 hours before death—yet death occurred, or (b) the basic experiments reported by Keppie et al[7] in which death of guinea pigs was only delayed if streptomycin was administered after a septicemic level of about 1×10^6 bacilli/milliliter was attained. The data of Keppie et al[7] have been presented in Figure 24 to show the relationship between the level of bacilli in the blood and time-to-death of both streptomycin-treated guinea pigs and the untreated controls. The parallel between the two human cases (and deaths) at Fort Detrick and the data of Keppie et al[7] is remarkable. In both the human and experimental cases, administration of antibiotic resulted in a relatively rapid clearance of bacilli from the blood, followed by a much slower clearance of bacilli in the organs and phagocytic cells. Toxin potentially continues to increase as long as bacilli are alive. The critical level of toxin, i.e., the threshold amount that will cause death, may be reached before or after antibiotic treatment is initiated. In the first case, as in the work of Keppie et al,[7] death is assured and treatment merely increases the time-to-death by removing, more or less, completely the source of toxin, i.e., live bacilli. Whether an antitoxin can influence the course of disease and prevent death in this circumstance remains to be determined, but it seems probable that there must be a time-concentration interaction to be considered. In the second case, at the time treatment is initiated, the critical concentration of toxin has not been reached, but the necessary increment is produced while bacilli remain alive in the body. In this case, antiserum would be expected to prevent death, provided antibiotic treatment is continued until disease is controlled. It seems reasonable to predict that specific antiserum should be used soon after antibiotics in order to neutralize (a) unbound toxin formed prior to the antibiotic administration, (b) that being produced by bacilli remaining alive, and (c) that released from lysing and killed by antibiotics and phagocytosis. Our evidence that toxin alone kills monkeys and can be neutralized by specific antiserum, although preliminary and uses crude toxin, support this view. If this view is correct, an antitoxin must be used in treatment of generalized anthrax and also the antibiotic of choice must be a bactericidal rather than a bacteriostatic type in order to reduce to a minimum toxin produced after treatment is initiated. Criticism of this view is that toxic amounts of protein may be thrown onto the host if bacilli are lysed (as with penicillin), but killed vegetative cells of B. anthracis have been notoriously nonantigenic.

We have suggested that challenge with a small dose, i.e., one that may or may not establish disease, is more hazardous than challenge with a large dose, i.e., one expected to certainly establish disease, because the responses resulting from the larger dose would be more uniform and predictable. Our preliminary observations on cholinesterase inhibition and on detection of
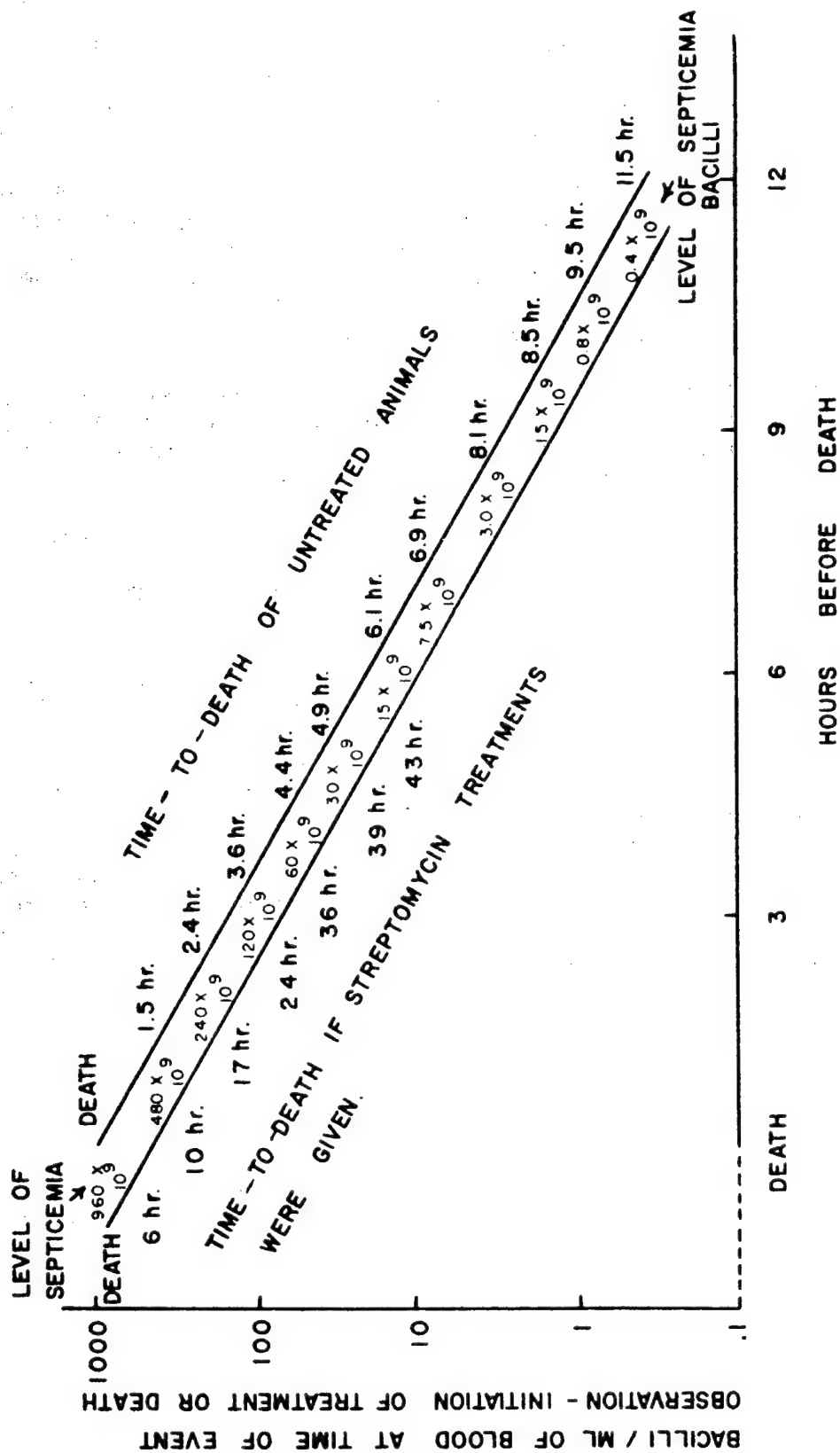
Figure 24. Relationship Between Level of Anthrax Bacteremia, Antibiotic Treatment, and Time-to-Death of the Guinea Pig. (Data from Keppie et al[7])

toxic products with tissue culture cell sheets, as well as model development, are encouraging in an otherwise sterile area. If detection were early enough, even present medical methods and concepts of treatment may well be adequate to allow positive control of such early diagnosed cases of this disease.

Felkner[34] has developed streptomycin-dependent strains. Various levels of dependency occur. The possibilities of using streptomycin-dependent strains for decreasing the hazard following inadvertent or deliberate challenge are interesting and need to be evaluated. If feasible, then the growth of the bacillus in the body could be controlled very readily, dependent on the level of streptomycin maintained.

In the immediately preceding paragraphs we have discussed challenge of man from the standpoint of what we have achieved technically. It seems equally appropriate to discuss what still remains to be done before serious consideration is given to challenging man deliberately.

All work is directed toward treatment. Research on pathogenesis and interactions of the course of disease needs to be extended to a point in which treatment becomes a science rather than medical art. The art as practiced up to now has unfortunately been proved to be inadequate.

The experiments that remain to be done prior to demonstrating that monkeys or chimpanzees can be cured of generalized anthrax established via aerosol challenge can be, in fact, are clearly visualized. The areas of experimentation are: (a) determining distribution of bacilli within tissue during both the accelerating growth curve after infection and the decelerating death curve after treatment is initiated; (b) determining the evaluation of immune sera for most favorable toxin neutralization and rapid clearance of bacilli in the tissue; (c) development of a drug therapy for toxin; (d) development of optimum treatment conditions, particularly in regard to antibiotics but also considering supplementary oxygen, fluids, and environmental control; (e) diagnostic tests to indicate early establishment of infection; and (f) development of a procedure to prevent secondary reinfection after treatment and cure.

The dose required to infect is virtually unimportant except in consideration of the residual spores left in the lungs for potential secondary infection after cure.

Knowledge of the quantitative distribution and changes of bacilli in the body as a whole and each of the tissues needs to be determined during both the build-up following dosage and the fall-off after treatment is initiated. This information is not available for any disease. Except for the observations of Keppie et al[7] almost nothing is known about number and distribution of anthrax bacilli from dosing to septicemia. Treatments that will rapidly reduce the bacterial count in both the blood and the fixed tissues are desired. The protection from antibiotic action given bacteria by phagocytosis following treatment with specific "spore" antiserum would determine the optimum time of administering antiserum, an interaction considered in the next paragraph.

Specific immune sera are a very complex mixture of chemicals that cause many general reactions, as well as the desired one of counteracting the pathogen or its products. The ideal antiserum for treatment of generalized anthrax would be a specific toxin-neutralizer without side-effects. The specific antiserum known as "spore" antiserum may be expected to increase phagocytosis of the vegetative cells. Although specific antiserum neutralizes toxin at least to some degree both *in vivo* and *in vitro*, we have given our reasons for believing that immunity due to protective antigen only or protective antigen plus live vaccine is not primarily toxin-neutralizing. Methods and plans for determining how 16 sera (the eight factorial combinations of the three toxin components with and without live vaccine) meet these criteria are given in Section IV, A. Other antigens, such as the spore wall, spore membrane, vegetative wall, and capsule might give interesting results. Purification of the gamma fraction from the selected antiserum would be expected to reduce the side-effects frequently observed when seroprophylaxis is used.

Seroprophylaxis is one form of treatment for the toxin. Drug therapy specific for anthrax toxin would be expected to have a different mode of action, probably counteracting the physiological effect of toxin, as well as being of a molecular size that would penetrate cellular membranes more readily than gamma globulin. The effect of toxin alone on rats, rabbits, and monkeys strongly suggests that anthrax toxin is a neuro-toxin, affecting the hypothalmus and/or adrenal glands (Section IV, B).

Treatment today depends entirely on antibiotics that are effective only against the bacillus. Serotherapy and drug therapy, if specific, would both neutralize toxin and counter its physiological effect. We believe that neutralization of the "free" toxin, i.e., not fixed in tissue, is possible by specific antitoxin antiserum and that the physiological effects of toxin may be counteracted by drug therapy. In addition, other treatments favoring the host should be used as oxygen and administration of fluids. An edema is characteristic of anthrax. Control of the edema should be considered, possibly cortisone administered a few hours after antibiotics. If drug and serotherapy are developed, the edematous phase of this disease may no longer be observed. A reasonable philosophy for optimum treatment may be (a) massive administration of the bactericidal types of antibiotic as early as possible (penicillin and/or streptomycin, not the bacteriostatic tetracycline drugs); (b) two to four hours later, administer antitoxin antisera to neutralize the "free" toxin; (c) at time a or b above, drugs specific for toxin would be given; and (d) other treatment would be given as appropriate. Since death from anthrax or anthrax toxin alone occurs suddenly, frequently after the host's condition appears to be improving, the supplementary aids should be given before toxic symptoms or distress occurs.

An early diagnosis for anthrax would decrease the hazard of challenge. Antibiotic treatment alone would then be adequate because treatment would be started before a critical level of toxin had been generated within the host. When considering only the problem of deliberate challenge, the

necessity of the diagnostic test being specific for anthrax can be ignored.
Two such tests have been described in Sections IV, C and IV, D. Only by
continuing research on anthrax will such tests be developed. It is probable
that the ability to detect and quantitate bacilli in the blood could be
made from 5 to 50 times more accurate by centrifugation, lysing of blood
cells, filtering, and observing the growth of micro-colonies. Identification
of anthrax by its bacteremial level has the inherent faults that significant
growth in the host has occurred and recognition of the bacillus is delayed
until colonial growth can occur, during which time, corresponding growth
will occur in the host.

Following an aerosol exposure, spores are depositied on the membranes
of the lung alveoli, but only a small percentage of the total number of
spores is removed in any unit of time, such as per day. Based on observa-
tions among survivors of aerosol tests, these residual spores are capable
of causing disease for at least 200 days in monkeys. Procedures that will
positively prevent delayed injection or reinfection must be developed. At
this time, two ways appear possible. A level of penicillin could be main-
tained that would destroy susceptible anthrax bacilli. This is not impos-
sible or unfeasible because several million humans now being treated for
other diseases are maintained in this way for months or years. The second
way would be to treat with penicillin at the end of the primary observation
period and simultaneously initiate immunization with protective antigen
(it is recognized that one of the other components of toxin may prove a
superior immunizing antigen when tests are finally conducted). Immunization
and penicillin will be continued until a serum titer of at least 1:8 is
developed, then live vaccine will be given. A week later the live vaccine
administration of penicillin can be removed and a second dose of live
vaccine given. The resistance to establishment of disease should be elevated
to a level preventing infection by residual spores.

To more fully complete the biological picture, several scientifically
worthwhile and intellectually stimulating areas of research need to be
explored. The information gathered would not advance the possibility of
determining the dose required to infect man but would be of basic interest.

1. Barnes[13] has reported that after aerosol exposure about 70 per cent
of the retained spores are found in the stomach within a few hours. It
would be desirable to know under what conditions infections through the
stomach might be expected, and thereby evaluate the importance of spores
deposited on the ciliated membranes of the lung and trachea.

2. Our work does not disagree with the observations of Ross[2] regarding
movement of spores from the lung alveoli into the lymph stream via motile
phagocytes. It would be of interest to know whether or not white blood
cells are absolutely essential to initiate infection. Techniques are avail-
able to almost completely block or destroy the phagocytic system so that
this experiment appears feasible.

3. We have developed a rather unique method for cannulation of the lymphatics in survival experimentation. Whether toxin is distributed primarily by the lymphatics and, whether toxin can be absorbed from the lungs and gut, needs to be determined.

4. Although the present group cannot obtain the desired information, attention is called to the need for observing the terminal concentration of bacilli/milliliter of blood in human cases. It is possible that American or English hospitals may have this information in old records, but quantitation of observations is not to be expected. This information might be obtained from new, current cases, but since anthrax cases occur very infrequently and hospital routine would need to be changed to secure satisfactory information, the probability is slight for obtaining the desired information. It is possible, however, to obtain this information if personnel could be sent to some of the epidemics that occur each year, principally in the Near East due to eating diseased, uncooked meat. After such exposure, cases occur for several days and the chance of obtaining the desired information is high enough to recommend this approach. It would be desirable at this time to establish procedures that would positively obtain maximum information for hospital cases. Procedures reported in this report form the basis of such a recommendation.

5. The final area of work that will be mentioned, related to this program, is that of lowering the dose required to infect from the aerosol. Our work and literature review in this area are reported in Section III, B. A reasonable objective would be a one-log, and quite possibly a two-log, reduction in dose. Several feasible ways may be mentioned.

a. Chemicals acting as a co-agent have been reported[14, 17, 18] and this area might be extended.

b. Since infection involves phagocytosis from the lung alveoli, two approaches appear feasible.

(1) The space could be made so that phagocytosis occurred more readily or avidly, as after coating with an antigen or irritant, or

(2) Strains resistant to destruction by the phagocyte could be selected, i.e., tolerant to lactic acid, lysozyme, or the anaerobic environment of the phagocyte.

c. It seems feasible to develop strains of increased toxin production or more toxigenic in action as a result of new interactions due to addition, substraction, or changed proportions of the several constituents of anthrax toxin.

     d.   Selection of a strain refractive to immunity due to protective antigen or antigens of vaccine strains in common use as the Sterne strain may be possible. Three strains, including V116, are refractive to 2.7 logs increase in resistance attributable to immunity by protective antigen. Selection in an _in vivo_ environment of immunized guinea pigs might be expected to move this value still higher. Use of cold-blooded hosts might be considered to select for new antigens. Transfer of antigens from other _Bacillus_ species also appears feasible, as both a transformation and transduction system has been developed for _B. anthracis_. It is to be noted, however, that the primary problem is, and remains, what is the dose required to infect man, not how can the dose be lowered, even though progress may be expected by implementing these and other approaches to this problem.

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APPENDIX

## METHOD FOR CANNULATION OF THE LYMPHATICS

If the hypothesis on pathogenesis of anthrax, given in the body of this memorandum, is true, then bacilli should appear in large numbers in the lymph before being observed in the blood as a septicemia, and this might be a way to detect anthrax infection before it enters the septicemia stage. Accordingly, procedures to cannulate the thoracic and right lymph ducts in survival experiments were developed. Four procedures were tried before a successful one was evolved. The first approach was in the cervical region, but cannulation of the lymphatic ducts failed because of inadequate working space and exposure of ducts. A second approach in which the chest was entered by transection of the clavical and first ribs was abandoned, because this procedure required artificial respiration and resulted in post-operative complications that prevented continuous collection of lymph. A third procedure, entering through the intercostal space, was unsuccessful because of inadequate exposure of ducts and working space, the need to enter both sides of the chest, and extensive post-operative complications.

A successful procedure was developed by bisecting the sternum and entering the superior mediastinum. This procedure allowed ample exposure of ducts and working space to cannulate both thoracic and right lymphatic ducts, minimum post-operative complications, and continuous collection of lymph. Only monkeys have been used in these experiments, but the procedure appears suitable for use in all cases where recovery of the animal is necessary following cannulation.

Since the rhesus monkey is quite sensitive to ether and the margin of safety is very narrow, the monkey is suitably anesthetized with pentabarbital sodium. The head, neck, arms, and chest are shaved, cleaned, and disinfected. The trachea is intubated, and the tube is connected to a positive oxygen system. Asepsis is maintained throughout the procedure. With the monkey properly draped, a mid-line skin incision is made over the sternum, extending from about five centimeters anterior to the manubrium, to about five centimeters caudal to the second ribs. The sternum is bisected from the manubrium to the caudal limit of the second ribs with an electric bone saw (Figure 1). The major pectoralis muscles, which originate on both sides of the sternum, provide an excellent guide. Care is taken to remain in the center of the sternum to avoid severing the internal mammary vessels located on both sides of the sternum. Normally, the pleural cavity is not entered; however, if it is entered inadvertently, no harm is caused because positive oxygen pressure is maintained throughout the procedure. A self-retaining retractor is used to retract the bisected sternum. The thoracic and common right lymphatic ducts are exposed by blunt dissection at their respective entrances into the blood stream at the junction of the internal and external jugular veins (Figure 2). Either duct can be cannulated first.
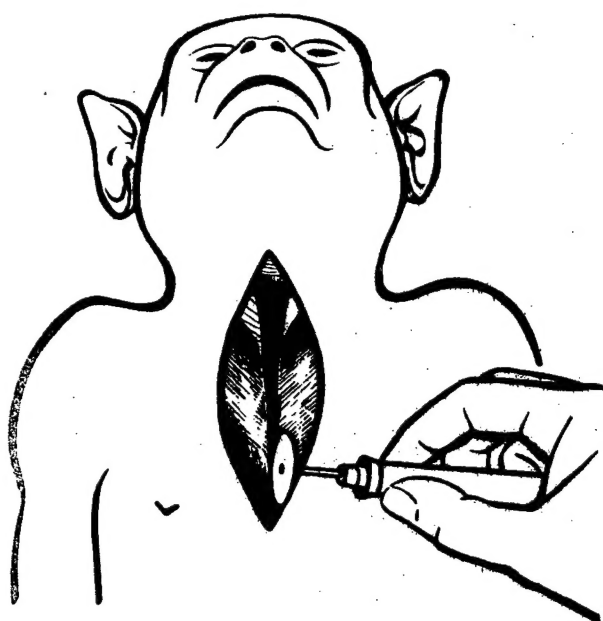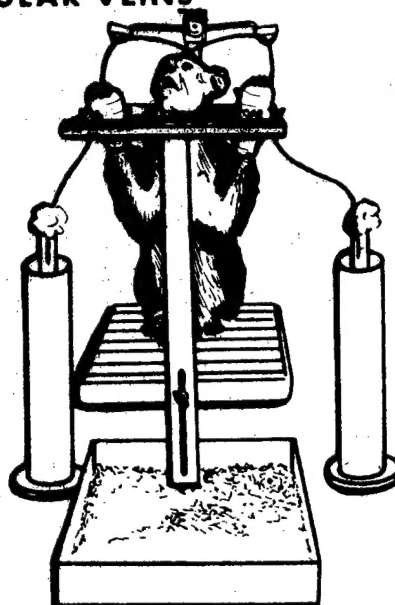
FIGURE 1—BISECTED STERNUM
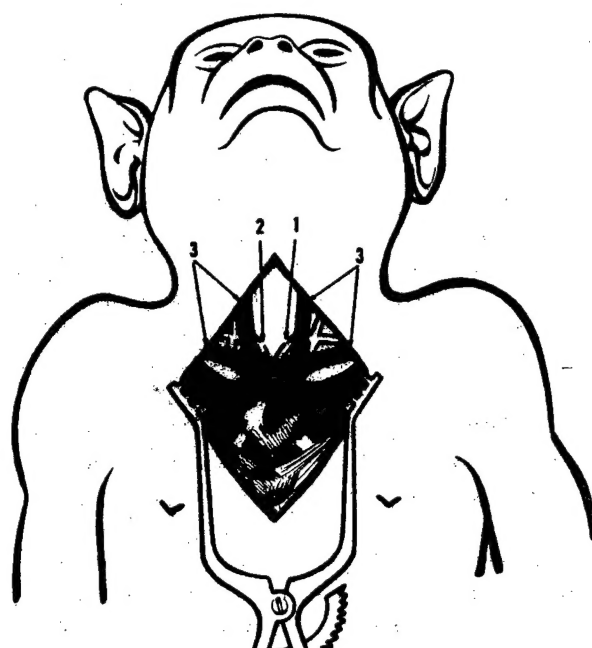
FIGURE 2—ISOLATED THORACIC AND RIGHT LYMPHATIC DUCTS
1. THORACIC DUCT 2. RIGHT DUCT
3. JUGULAR VEINS
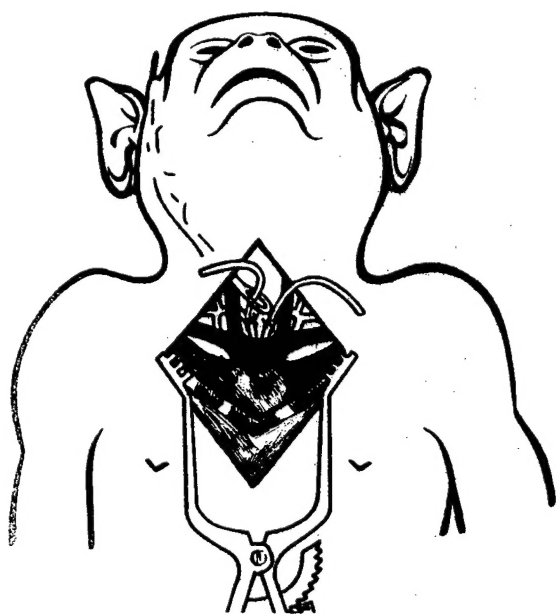
FIGURE 3—CANNULATED THORACIC AND RIGHT LYMPHATIC DUCTS. A FLEXIBLE EYE PROBE IS SHOWN UNDER THE SKIN OF THE NECK AND HEAD.

FIGURE 4—THE MONKEY IS PLACED IN THE CHAIR POST OPERATIVE

The duct is ligated at its entrance into the blood system, and the ends of the ligature are left long for later use in securing the cannula. A second ligature is placed loosely around the duct about three to four centimeters from the first. The jugular vein is retracted medially or laterally to facilitate cannulation. A small longitudinal incision is made in the duct just proximal to the first ligature with the cutting edge of a hypodermic needle or a suture needle. A sharp pointed probe, bent to approximately a right angle, is inserted into the duct in a retrograde direction to keep the incision open. The probe is held in the left hand, and the beveled end of sterile polyethylene tubing filled with diluted heparin is held with smooth forceps in the right hand. The tip of the tube is placed into the duct. The probe is slowly pulled out and the tube is inserted as far as possible. (If the flow of lymph is great enough, the incision will remain open and the probe, therefore, need not be used.) The tube can be inserted with slight traction on the ends of the ligature used to tie off the duct. Usually, a valve is encountered in the duct and, in order to get lymph flow, the tube must be inserted with slight rotation beyond this valve. When cannulation is successful, heparin will immediately flow from the distal end of the tube. The tube is secured in the duct with the previously placed loose ligature and is further secured with the ligature originally used to tie off the duct. Both ducts are cannulated before attempts are made to bring them out of the chest (Figure 3). At the same time, the heart is catherized by cannulation of the jugular vein, i.e., the cannula is passed through an incision of the vein until the heart is reached.

The positions and directions of each duct vary slightly; therefore, the cannulae are brought out as near as possible in line with the direction of the duct and, in such manner, that the orientation of the cannula is proper to minimize traction and angulation of the tube and duct when the monkey changes position, or adjacent organs are displaced by respiratory movements. A probe is used to puncture the cleido-mastoid muscle at the point that will best provide good alignment of tube and duct. The tube is pulled through the puncture and then secured to the muscle to prevent slipping and dislodging when traction is applied. In a straight line leading from this point, a blunt flexible probe is used to tunnel under the skin of the neck and head; the probe is directed anteriorly to the ear until the scalp is reached (Figure 4). An incision is made over the point of the probe to free it. The tip of the cannula is placed in the eye of the probe, and the probe is pulled through the incision in the scalp bringing the cannula through the tunnel under the skin. Each cannula is brought out in the same fashion on its respective side of the head. The venous cannula is also brought out at this time.

The retractor is removed from the wound, and two or three sutures of No. 28 gauge stainless steel wire are used to approximate the bisected sternum. No. 000 plain catgut is used to approximate the loose connective tissue, and the skin is closed with either stainless steel wire or skin clamps. Antibacterial ointment is applied to the wounds to prevent post-operative infection.

Essential after-care includes placing the monkey in a simple holding chair designed to allow maximum freedom of the body (Figure 4). The chair consists of a base plate of stainless steel to which is attached a stainless steel rod in a vertical position. To the rod is attached a plexiglass plate with holes provided to secure the head and hands and a stainless steel rack for supporting the haunches and feet. The plexiglass plate and rack are adjustable to accomodate various-sized monkeys. This chair permits complete freedom from the chest down and allows sitting and partial standing indefinitely without ill-effect to the animal. Maximum freedom is significant, as lymph flow largely depends upon body movement. Lymph flows continuously and is collected by inserting the cannula through a rubber stopper into a test tube. The rate of flow approximates 0.5 milliliter/pound of monkey-hour. The venous cannula is connected to a hypodermic needle that is attached to a three-way stop-cock secured to a clamp on a vertical rod. The second arm of the stop-cock is attached to sterile, protein hydrolysate in five per cent dextrose. Blood samples may be taken by a syringe placed in the third arm of the stop-cock. The cannula is back-rinsed with protein hydrolysate to prevent clotting of blood in the cannula. Protein hydrolysate in five per cent dextrose is given intravenously at regular intervals during the post-operative period to prevent hypoproteinemia.

Factors favorably affecting lymph flow were short operation time, avoidance of unnecessary damage to the region drained by the lymphatics, proper alignment of tube and duct to prevent angulation when the monkey changes positions, and using polyethylene tubing long enough to hang 1 to $1\frac{1}{2}$ feet below the point of cannulation to provide a siphoning effect. The latter is very significant, by virtue of the vertical position in which the monkey is placed, which necessitates more hydrostatic pressure to maintain lymph flow as opposed to a horizontal position. It was also found that, in order to get a sustained lymph flow, No. 50 tubing (inside diameter, 0.023 inch and outside diameter, 0.034 inch) was essential, and only monkeys over 13 pounds possessed lymphatics that accomodated that size of tubing.

There is no apparent post-operative pain, and the monkeys recover from the surgery rapidly and without complication. The only difficulty encountered was with cases that had pre-existing organic conditions, severe lung mite infestation, etc., and those that were obviously poor surgical risks. In more than 50 per cent of the rhesus monkeys used, the common right lymphatic duct did not enter the blood stream at the junction of the internal and external jugular veins. In some animals, this duct could not be identified at all, probably because it was situated too deeply in the chest to be isolated by probing. In other animals, it was identified either at the junction of the subclavian and external jugular veins or entering directly into the great veins at some point anterior to the heart. When only the thoracic lymphatic duct was identified, we normally cannulated it and used the animals for experimentation to obtain information on the cutaneous, intraperitoneal, and intravenous routes of challenge. Although Evans' blue

dye was used initially to explore and map the lymphatic system draining the lung area, we did not use it except when the animal was to be sacrificed for this purpose, as its use might interfere with the course of the disease.

Lymph flow from the thoracic and right lymphatic ducts was maintained for several days, which allowed study of the pathogenesis of anthrax subsequent to aerosol challenge, as well as via other portals of entry.